



**Liliana Isabel Moreira
Costa**

**Avaliação do efeito da suplementação com
colesterol na hipertensão arterial pulmonar
experimental**

**Evaluation of the effect of cholesterol
supplementation on experimental pulmonary arterial
hypertension**



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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, com especialização em Bioquímica Clínica, realizada sob a orientação científica da Doutora Rita Marisa Nogueira Ferreira, Investigadora Auxiliar do Departamento de Cirurgia e Fisiologia da Faculdade de Medicina da Universidade do Porto; do Doutor André Pedro Leite Martins Lourenço, Professor Auxiliar do Departamento de Cirurgia e Fisiologia da Faculdade de Medicina da Universidade do Porto; e da Doutora Maria do Rosário Gonçalves dos Reis Marques Domingues, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro.

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Dedico este trabalho à minha família.

o júri

presidente

Prof. Doutor Francisco Manuel Lemos Amado

Professor associado com agregação do Departamento de Química da Universidade de Aveiro

Prof. Doutor Daniel Moreira Gonçalves

Investigador do Centro de Investigação em Atividade Física, Saúde e Lazer da Faculdade de Desporto da Universidade do Porto

Doutora Rita Marisa Nogueira Ferreira

Investigadora auxiliar do Departamento de Cirurgia e Fisiologia da Faculdade de Medicina da Universidade do Porto

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palavras-chave

caquexia cardíaca, insuficiência cardíaca, colesterol, hipótese endotoxina-lipoproteína

resumo

A caquexia cardíaca (CC) é uma complicação da insuficiência cardíaca (IC), caracterizada pela perda de peso involuntária, independente do consumo alimentar e associada a um mau prognóstico. Devido à falta de estratégias terapêuticas, é necessária mais investigação sobre a complexa fisiopatologia da CC, de forma a testar novas abordagens preventivas e terapêuticas. O paradoxo do colesterol baseia-se em estudos que associam baixos níveis séricos de colesterol total e de lipoproteína de baixa densidade a um pior prognóstico na IC. Considerando a hipótese da endotoxina-lipoproteína, que sugere que elevados níveis de colesterol e lipoproteínas possam atenuar a ativação imunológica associada à CC, admitimos a hipótese de que o aumento do consumo de colesterol na dieta poderia melhorar a IC e a CC na hipertensão arterial pulmonar (HAP) experimental. Este estudo teve como objetivo testar a hipótese da endotoxina-lipoproteína *in vivo*, pela avaliação dos efeitos funcionais e moleculares de uma dieta suplementada com colesterol no modelo animal de HAP induzido pela administração de monocrotalina (MCT). Ratos *Wistar Han* foram injetados com MCT (60 mg/kg) ou com um volume igual de veículo e, após cinco dias, os ratos injetados com MCT foram aleatoriamente repartidos para consumirem uma dieta normal ou uma dieta suplementada com colesterol (2 % de colesterol e 0.25 % de ácido cílico). Entre o 25º e 30º dia, os animais foram submetidos a uma avaliação ecocardiográfica e hemodinâmica. Foram determinadas a evolução do peso corporal, o pico de consumo de oxigénio, a área de secção transversal dos cardiomiócitos e o espessamento da parede de arteríolas pulmonares. A concentração plasmática de colesterol total, lipoproteína de alta densidade (HDL-C), colesterol não-HDL, triglicerídeos, fator de necrose tumoral alfa (TNF- α) e endotoxina LPS foram também determinadas. Os resultados demonstraram que a MCT induziu efetivamente o desenvolvimento de HAP, hipertrofia e insuficiência do ventrículo direito, acompanhado pela redução significativa do peso corporal, associado com a CC. A suplementação da dieta com colesterol induziu um aumento significativo da concentração plasmática de colesterol, HDL-C e colesterol não-HDL, um aumento da massa do fígado e da área de secção transversal dos cardiomiócitos do ventrículo esquerdo. Verificámos uma tendência para a redução dos níveis de TNF- α e endotoxina LPS, o que sugere que um aumento dos níveis circulantes de lipoproteínas pode reduzir a ativação inflamatória induzida pela endotoxina LPS. Assim, é necessária mais investigação acerca da suplementação com colesterol na CC, de forma a clarificar estes efeitos.

keywords

cardiac cachexia, heart failure, cholesterol, endotoxin-lipoprotein hypothesis

abstract

Cardiac cachexia (CC) is a serious complication of heart failure (HF), characterized by involuntary weight loss, independent of food intake and associated with poor prognosis. Given the lack of therapeutic strategies for CC, further research is needed to explore its complex pathophysiology and to test new preventive and therapeutic approaches. The cholesterol paradox is based on reports that low total cholesterol and low-density lipoprotein serum levels worsen prognosis in HF. Considering the endotoxin-lipoprotein hypothesis, which states that higher circulating levels of cholesterol and lipoproteins can attenuate the CC-related immune activation, we hypothesized that enhancing cholesterol intake would ameliorate HF and CC in experimental pulmonary arterial hypertension (PAH). This study aimed to test the endotoxin-lipoprotein hypothesis *in vivo* by evaluating functional and molecular effects of a cholesterol supplemented diet in monocrotaline (MCT)-induced rat PAH. *Wistar Han* rats were injected with MCT (60 mg/kg) or an equal volume of vehicle and, five days after, MCT-injected rats were randomly allocated to consume either normal diet or a cholesterol supplemented diet (cholesterol 2 % and cholic acid 0.25 %). Between the 25th and 30th day, animals underwent echocardiographic and haemodynamic evaluation. We assessed body weight (BW) evolution, peak of oxygen consumption, cardiomyocyte cross-sectional area and pulmonary arterioles wall-thickness. Plasma concentration of total cholesterol, high density lipoprotein-cholesterol (HDL-C), non-HDL cholesterol, triglycerides, tumour necrosis factor alpha (TNF- α) and endotoxin LPS was also determined. The results showed that MCT effectively induced the development of PAH and right ventricle hypertrophy and failure, accompanied by a significant reduction of BW, which is related with CC. Cholesterol supplemented diet induced a significant increase of plasma total cholesterol, HDL-C and non-HDL cholesterol concentration, liver weight and left ventricle cardiomyocyte cross-sectional area. We found also a trend towards lower plasma levels of TNF- α and endotoxin LPS, suggesting that the higher lipoprotein content might reduce the inflammatory activation induced by endotoxin LPS. Further research is needed regarding cholesterol supplementation in CC, in order to clarify these effects.

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peak myocardial systolic velocity near the tricuspid annulus; sc, subcutaneous; SP, systolic pressure; τ , time-constant of isovolumic relaxation by Weiss formula; TAPSE, tricuspid annular plane systolic excursion; TL, tibial length; TNF- α , tumour necrosis factor alpha; VO₂, oxygen consumption; VVC, ventricular-vascular coupling. 29

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Abbreviations

ACE	Angiotensin-converting enzyme
ActRIIB	Activin receptor type 2B
AET	Aerobic exercise training
ALK	Activin like kinase
Ang II	Angiotensin II
ANP	A-type natriuretic peptide
AR	Androgen receptor
AT₁	Angiotensin II receptor type 1
ATP	Adenosine triphosphate
β-AR	β-adrenergic receptor
BMI	Body mass index
BNP	B-type natriuretic peptide
BSA	Body surface area
BW	Body weight
CAD	Coronary artery disease
CC	Cardiac cachexia
CD	Cholesterol diet
CD14	Cluster of differentiation 14
cGMP	Cyclic guanosine monophosphate
CI	Cardiac index
CL	Cycle length
CO	Cardiac output
CoQ10	Coenzyme Q10
COPD	Chronic obstructive pulmonary disease
CSQ	Calsequestrin
DP	Diastolic pressure
DS	Dahl salt-sensitive
EDP	End-diastolic pressure
EDV	End-diastolic volume
EF	Ejection fraction
ELISA	Enzyme-linked immunosorbent assay

ESC	European Society of Cardiology
ESP	End-systolic pressure
FBXO32	F-box only protein 32
FOXO	Forkhead box O
GH	Growth hormone
GPx4	Glutathione peroxidase 4
GW	<i>Gastrocnemius</i> muscle weight
HDL	High density lipoprotein
HDL-C	High density lipoprotein - cholesterol
HF	Heart failure
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HR	Heart rate
IGF-1	Insulin-like growth factor-1
IGFBP4	Insulin-like growth factor binding protein-4
IGF1R	Insulin-like growth factor-1 receptor
IL	Interleukin
IR	Insulin receptor
IRS1	Insulin receptor substrate 1
IVC	Inferior vena cava
IVS	Interventricular septum
LAD	Left anterior descending
LAL	<i>Limulus</i> amebocyte lysate
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein - cholesterol
LPB	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
LV	Left ventricle
MAPK	Mitogen-activated protein kinase
MCT	Monocrotaline
MCTP	Monocrotaline pyrrole
MP	Mean pressure
mRNA	Messenger ribonucleic acid

mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
ND	Normal diet
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
NP	Natriuretic peptide
NPR-A	Natriuretic peptide receptor A
NPY	Neuropeptide Y
PAAT	Pulmonary artery acceleration time
PAH	Pulmonary arterial hypertension
PGFW	Perigonadal fat weight
PH	Pulmonary hypertension
PI3K	Phosphoinositide 3-kinase
PKG	Protein kinase G
PP	Pulse pressure
PRFW	Perirenal fat weight
PUFA	Polyunsaturated fatty acid
PV	Pressure-volume
RAA	Right atrial area
RV	Right ventricle
SEM	Standard error of mean
SP	Systolic pressure
TAC	Transverse aortic constriction
TAPSE	Tricuspid annular plane systolic excursion
TG	Triglycerides
TL	Tibial length
TNF-α	Tumour necrosis factor alpha
TRIM63	Tripartite motif containing 63
TWL	Total workload
UPS	Ubiquitin-proteasome system
VLDL	Very low density lipoprotein
VVC	Ventricular-vascular coupling

WD Western-type diet

1. Introduction

Cachexia, a syndrome characterized by involuntary weight loss independent of food intake, accompanies several acute and chronic diseases and entails a poor prognosis [1–3]. It is mostly associated with cancer, but also with end-stage chronic heart failure (HF), which is a major public health problem and one of the most common causes of death in Western countries [4,5]. HF-associated cachexia, one of HF's most dreaded complications, is termed cardiac cachexia (CC). It associates with decreased exercise capacity, impaired wellbeing and survival [6]. It is characterized by an imbalance in the activation of anabolic and/or catabolic pathways, that results from alterations in immunological, metabolic and neurohormonal processes [5,7]. However, the molecular mechanisms underlying CC are still poorly understood [8]. Recent studies have tackled the development of animal models, in order to understand the molecular mechanisms of this condition and also to find targets for efficient therapies [9]. Nevertheless, currently the mainstay of CC treatment is simple management of the underlying disease and associated comorbidities. Further studies are needed to track new preventive and therapeutic approaches in order to counteract CC-related weight loss and to increase muscle strength and exercise capacity in HF patients [10].

Hypercholesterolemia is an established risk factor for the development of HF. Paradoxically, low serum levels of total cholesterol and low density lipoprotein cholesterol (LDL-C) have been reported as an adverse prognostic marker in patients with HF [11,12]. This “reverse epidemiology” phenomenon, an unexpected association between improved survival and classical cardiovascular risk factors, including higher total cholesterol plasma levels, body weight, or systolic blood pressure, remains unexplained [13]. Several epidemiological and biological hypotheses have been raised. One of the most acclaimed biological hypothesis is the endotoxin-lipoprotein hypothesis [14]. As an introductory note we briefly review the main features of CC, including its definition, pathophysiology and treatment, the cholesterol paradox in HF and the hypotheses raised to explain it.

2. Cardiac cachexia

Cachexia is one of the most visible and devastating consequences of human disease, severe enough to constitute a public health problem [1]. It was first described by Hippocrates (about 460-377 BC) [15] ‘the flesh is consumed and becomes water (...), the abdomen fills with water, the feet and legs swell, the shoulders, clavicles, chest and thighs melt away (...). This illness is fatal’. Hippocrates recognized the severely impaired prognosis of this syndrome, as he describes a patient who is just ‘skin and bones’ [16]. The term *cachexia* is of Greek origin, derived from *kakós* (ie, bad) and *hexis* (ie, condition), literally meaning bad condition. It is characterized by a state of involuntary weight loss with pathologic wasting of muscle, with or without loss of bone mineral density and fat tissue, which is independent of food intake [2,3]. One of the most common misconceptions related to cachexia is that one of the underlying causes of cachexia is anorexia, i.e. loss of appetite. Although anorexia is certainly a feature of the diseases leading to the development of cachexia, the condition of anorexia alone cannot explain the metabolic changes observed on a cachectic patient [3]. Additionally, terms as “cachexia”, “anorexia”, “malnutrition” and “sarcopenia” are frequently used as synonyms although they relate to different conditions [3,17]. In contrast to cachexia, the weight loss presented in malnutrition and anorexia results from the decrease of fat mass for energy yield to balance the low caloric and food intake, while muscle mass is mostly spared, and all the symptoms and signs can be reversed by food supply [18]. Furthermore, sarcopenia is described as the age associated process of muscle wasting, which may not lead to a significant body weight loss, since loss of muscle and increase in fat mass are frequently balanced [3].

Several acute and chronic diseases, particularly in advanced stages, are associated with cachexia, including infectious diseases, such as HIV/AIDS, malaria, tuberculosis, cancer, HF, chronic obstructive pulmonary disease (COPD), chronic kidney disease, rheumatoid arthritis, and cystic fibrosis [2,17]. Cachexia is not only related with poor prognosis, but also associated with an unfavourable response to drug treatment and poor quality of life [3,19]. It is mostly concomitant with cancer, where the prevalence can reach 50-80% in advanced malignant cancer, but also with end-stage HF, where its prevalence ranges 5-15% and rises nearly exponentially with age [4,19].

2.1 Definition of cardiac cachexia

The earliest written documentation of the term CC comes from a French physician, Charles Mauriac [21], in 1860, as he wrote ‘commonly observed secondary phenomenon in patients affected with diseases of the heart (...) a peculiar state of cachexia which is (...) conventionally designated cardiac cachexia’. Apart from this report, CC as a syndrome has not been studied in much detail by clinical scientists for many years, since most patients with HF would not reach the state of chronicity for cachexia to develop. In comparison to cachexia related tuberculosis, malignancies or uncontrolled metabolic diseases, CC was considered as a rather rare condition [16,19].

A few decades ago, CC has been recognized as a serious complication of HF, associated with a decrease in exercise capacity, clinical wellbeing and survival [6]. However, the underlying mechanisms of CC are not well understood and there is no universal agreement upon a definition [8,19]. In 1999, Anker and Coats reported that CC should be considered when weight loss was superior to 7.5% of the previous normal weight, observed in patients with HF with, at least, 6 months duration and without signs of other primary cachectic states (such as cancer, thyroid disease or severe liver disease) [1]. In 2003, this definition was adjusted from the Studies of Left Ventricular Dysfunction database and CC was considered as a weight loss superior to 6%, over a period of at least 6 months [22]. The most recent consensus for the definition of cachexia is from the Cachexia Consensus Working Group [8], proposed in 2008. This definition for the diagnosis of cachexia in adults required that patients should have an underlying disease and body weight loss $\geq 5\%$ in ≤ 12 months, or body mass index (BMI) $< 20 \text{ kg/m}^2$, and at least three of the following five criteria should be observed: decreased muscle strength, fatigue, anorexia, low fat-free mass index or abnormal biochemistry (increased inflammatory markers, anaemia and low serum albumin levels) [8]. However, the precise criteria to define cachexia still vary among research groups, mostly in terms of the body weight loss cut-off point [4,19]. A recent study used an old definition, namely unintentional non-oedematous weight loss of $> 5\%$, over at least 6 months. By applying this definition, cachexia was found in 10.5% of HF patients [23]. In contrast, another study used the most recent definition and reported that 16% of HF patients were considered as cachectic [24]. Therefore, the use of the new cachexia definition and, more precisely, the addition of

criteria on top of obligatory weight loss has major implications on cachexia prevalence report and diagnosis [7,19].

2.2 Pathophysiology of cardiac cachexia

The HF-related mechanisms that result in CC are still poorly comprehended. In 1964, Pittman and Cohen [25] pointed cellular hypoxia as a leading pathogenic factor, inducing catabolism and reducing anabolism. The overall net catabolic dominance in HF accounts for a continuous peripheral loss of skeletal muscle, termed as muscle wasting, with or without loss of fat tissue later in the disease. This pathological condition of HF is often associated with CC, contributing to exercise intolerance and poor prognosis [26]. However, cachexia and muscle wasting terms must not be used interchangeably. The hallmark symptom of cachexia is weight loss, highly predictive of morbidity and mortality, whereas muscle wasting *per se* means loss of muscle mass without weight loss, because of the replacement of functional muscle by adipocytes and fat or other inactive tissue [27,28].

The catabolic/anabolic imbalance in CC results from alterations in several mediators involved in immunological, metabolic and neurohormonal processes [5,7]. These include pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF- α), interleukin (IL)-1 and IL-6, epinephrine and norepinephrine, angiotensin II (Ang II), aldosterone and myostatin, which promote protein degradation and stimulate energy production, and anabolic factors such as growth hormone (GH), ghrelin, insulin-like growth factor 1 (IGF-1), insulin, leptin and adiponectin, that regulate protein synthesis (Table 1) [29].

Table 1. Variation of the circulating (plasma and serum) levels of catabolic and anabolic factors in chronic heart failure (HF) patients, with and without cardiac cachexia (CC).

	Mediator	HF	HF with CC	References
Catabolic factors	TNF- α	\leftrightarrow/\uparrow	\leftrightarrow/\uparrow	[30–35]
	IL-1 β	\leftrightarrow	\leftrightarrow	[32]
	IL-6	\uparrow	\leftrightarrow/\uparrow	[32,33,35]
	Epinephrine	\leftrightarrow/\uparrow	\leftrightarrow/\uparrow	[30,32–34,36]
	Norepinephrine	\leftrightarrow/\uparrow	\leftrightarrow/\uparrow	[30,32–34,36]
	Angiotensin II	\leftrightarrow	\uparrow	[33]
	Aldosterone	\leftrightarrow	\uparrow	[30,33]
	ANP	\uparrow	\leftrightarrow/\uparrow	[33,34,36]
	BNP	\uparrow	\leftrightarrow/\uparrow	[33–36]
Anabolic factors	GH	\leftrightarrow	\uparrow	[30,33,37]
	Ghrelin	\leftrightarrow	\uparrow	[33,38]
	IGF-1	\leftrightarrow	$\leftrightarrow/\downarrow$	[30,33,37]
	Insulin	\leftrightarrow/\uparrow	\leftrightarrow	[30,32,33,39]
	Leptin	\leftrightarrow/\uparrow	$\leftrightarrow/\downarrow$	[35,39,40]
	Adiponectin	\leftrightarrow/\uparrow	\uparrow	[24,35,41]

Legend: \uparrow increase, \downarrow decrease, \leftrightarrow no variation. HF: heart failure; CC: cardiac cachexia; TNF- α : tumour necrosis factor- α ; IL-1 β : interleukin-1 β ; IL-6: interleukin-6; ANP: A-type natriuretic peptide; BNP: B-type natriuretic peptide; GH: growth hormone; IGF-1: insulin-like growth factor-1

2.2.1 Immune activation

Activation of inflammatory and immune pathways plays an important role in all forms of cachexia. Since the initial observation by Levine *et al.* [42], in 1990, several studies have demonstrated elevated circulating pro-inflammatory cytokines levels in HF patients, such as TNF- α , IL-1 and IL-6, as well as several chemokines, e.g., monocyte chemoattractant peptide-1, IL-8, and macrophage inflammatory protein-1 α [7,43,44]. When pro-inflammatory cytokines are overproduced, within the myocardium or in extramyocardial tissue, they can spread through peripheral circulation and activate the immune system [45]. Furthermore, the rise of these inflammatory mediators seems to be combined with inadequately raised or even decreased levels of anti-inflammatory mediators, namely IL-10 and transforming growth factor beta-1 [7,46,47].

Since the cause of immune activation is still uncertain, some hypothesis have been raised to explain it [5,7]. The first hypothesis assumes that the myocardium itself is the main source of pro-inflammatory cytokines, since the failing myocardium, due to ischemia or mechanical stress, is capable of producing TNF- α [48,49]. Indeed, TNF- α levels in the coronary sinus are increased compared with the aortic root, supporting that elevated plasma TNF- α is partly derived from the failing heart. However, the myocardial production of cytokines is rather a localized phenomenon [50]. The majority of pro-inflammatory mediators present in the systemic circulation are presumed to be secreted by circulating immune cells and the direct stimuli triggering their activation are still unknown [47,51]. The second hypothesis, known as endotoxin hypothesis, proposes that tissue hypoxia may be the primary stimulus for increased TNF- α production in patients with HF [52]. It is assumed that bowel wall oedema and ischemia, resulting from venous congestion, are responsible for an augmented intestinal translocation of bacterial endotoxin (lipopolysaccharide, LPS) into the systemic circulation, and subsequent activation of the circulating immune cells [53]. In the circulation, LPS is bound to a serum protein, termed lipopolysaccharide-binding protein (LBP). The LPS-LBP complex can interact with cluster of differentiation 14 (CD14) membrane protein and Toll-like signalling receptors activating a signalling cascade that leads to increased cytokine production [54]. Supporting this hypothesis, it has been reported that monocytes, one of the most important source for circulating TNF- α and IL-1 β , from HF patients, showed an increased TNF- α release, when stimulated with LPS, when compared to monocytes from patients without HF [53,55,56]. Additionally, another proposed theory assumes that the immune activation seen in HF patients is a consequence of the long-term neurohormonal overactivation and exaggerated stimulation of the sympathetic nervous system and that the mechanism triggering inflammatory processes is secondary to the central suppression of parasympathetic nervous system [5,51].

The pro-inflammatory cytokine TNF- α is suggested as a common mediator in all forms of cachexia. It activates the ubiquitin-proteasome system (UPS), autophagy mechanisms and apoptosis pathways, in skeletal muscle and other tissues, thus maintaining the wasting process in CC (Figure 1) [18,19]. The UPS involves cytokine-induced activation of Nuclear Factor-kappa B (NF- κ B) signalling in skeletal muscle. Depending on the upstream triggers, this pathway activation may modulate apoptosis, inflammation and

differentiation. Since $\text{TNF-}\alpha$ is a classical activator of $\text{NF-}\kappa\text{B}$ signalling, it is responsible for the induction of muscle atrophy in CC, as it up-regulates the transcription of members of UPS. This up-regulation can be mediated as well by forkhead box O (FOXO) transcription factors which regulate the expression of vital components of UPS [57]. Proteins to be degraded by this mechanism are first conjugated to multiple molecules of ubiquitin (ubiquitin activating enzyme E1, ubiquitin conjugating enzyme E2 and ubiquitin ligase E3) and then degraded in the 26S proteasome complex in an ATP-dependent process [58]. Since the proteasome is not able to degrade intact myofibrils [3], actin and myosin are first released by the action of proteolytic pathways, such as calcium/calpain-dependent proteolytic pathways [57,58].

$\text{TNF-}\alpha$ induces the cytosolic release of $\text{NF-}\kappa\text{B}$ from its inhibitory proteins $\text{I}\kappa\text{B}$, allowing the translocation of $\text{NF-}\kappa\text{B}$ into the nucleus and subsequent transcription of proteolytic pathway UPS components, such as F-box only protein 32 (FBXO32, also known as MAFBX or atrogin 1) and tripartite motif containing 63 (TRIM63, also known as MURF1) (Figure 1) [59]. These are E3 ubiquitin ligases specifically required for muscle atrophy, since FBXO32 inhibits factors associated with protein synthesis, such as the eukaryotic translation initiation factor eIF3-f [60], and TRIM63 targets myofibrillar proteins [61]. Nevertheless, $\text{TNF-}\alpha$ signalling through the mitogen-activated protein kinase (MAPK) p38 might also increase FBXO32 [62].

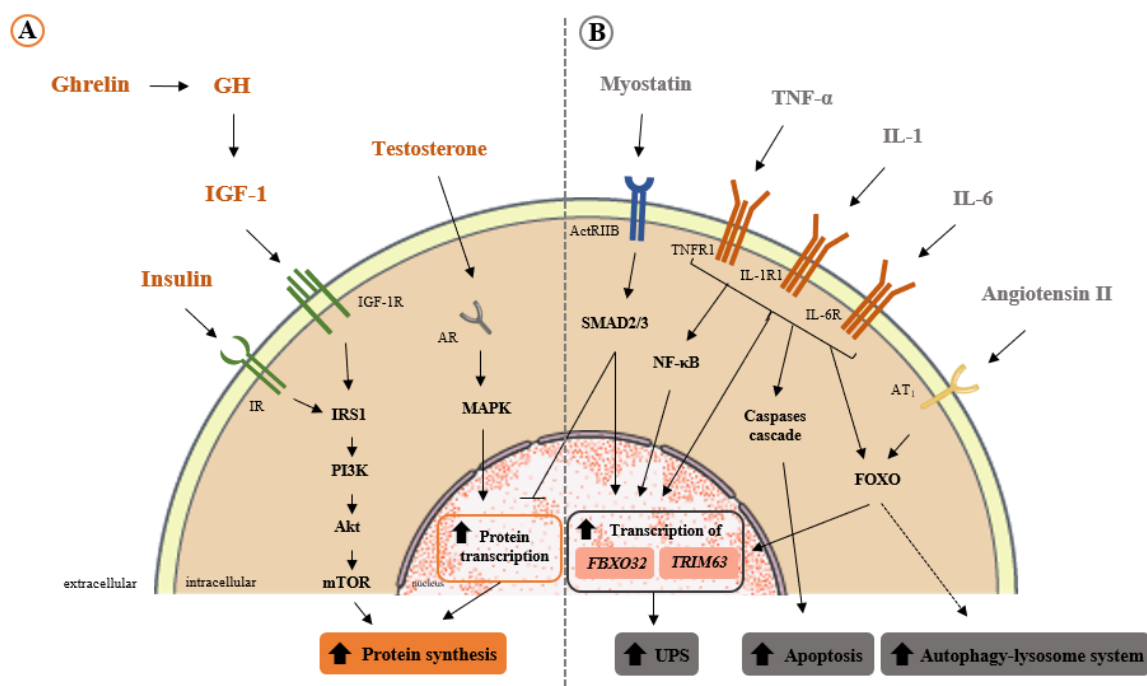


Fig. 1. Overview of signalling pathways involved in muscle wasting in heart failure. A) Anabolic pathways embrace the stimulation of growth hormone (GH) by ghrelin. GH induces secretion of insulin-like growth factor 1 (IGF-1), which binds, like insulin, to the IGF-1 receptor (IGF1R) and the insulin receptor (IR). The Akt-pathway is activated *via* insulin receptor substrate 1 (IRS1) and phosphoinositide 3-kinase (PI3K). Akt activates the serine/threonine protein kinase mammalian target of rapamycin (mTOR), which stimulates protein synthesis. Testosterone binds to cytoplasmic androgen receptors (AR) and stimulates protein transcription *via* mitogen-activated protein kinases (MAPK). B) Catabolic pathways activate three main protein degradation systems: the ubiquitin-proteasome system (UPS) by FBXO32 (also known as MAFBX) and TRIM63 (also known as MURF1) expression; apoptosis, initiated by caspases; and the autophagy-lysosome system, mediated by forkhead box protein O (FOXO) transcription factors. Myostatin binds to activin receptor type 2B (ActRIIB) activating transcription factors of the Smad family. Smad2 and Smad3 inhibit protein transcription and stimulate protein degradation *via* UPS. Pro-inflammatory cytokines, namely tumour necrosis factor- α (TNF- α), interleukins 1 and 6 activate not only their respective receptors, but also nuclear factor κ B (NF- κ B) and FOXO. Angiotensin II binds to angiotensin II receptor type 1 (AT₁) and activates FOXO as well. Anabolic mediators are presented in orange and catabolic ones in grey. The dashed lines indicate an indirect action. Adapted from [19].

The high circulating levels of TNF- α promote a rearrangement of the cytoskeleton of endothelial cells and an increase of albumin and water permeability, resulting in the development of endothelial dysfunction. The binding of TNF- α to its receptor induces caspases activation and, consequently, apoptosis signalling [63]. A long-term effect of increased TNF- α concentrations is thought to be the reduction of peripheral blood flow in HF patients, by its effect on the decrease of the vasodilator endothelial nitric oxide (NO) synthase mRNA in vascular endothelial cells [64,65].

The pro-inflammatory cytokines IL-1 and IL-6 also play an important role in CC. IL-1 induces the uncoupling of the β -adrenergic receptor (β -AR) from the adenylyl cyclase and from the L-type calcium channels, without affecting the β -AR density or the affinity for its ligands. Thus, it can inhibit cardiac myocyte β -adrenergic responsiveness, leading to a negative effect on myocardial contractility [66,67]. Increased circulating levels of IL-6 in HF patients are associated with a higher severity of the disease and poorer left ventricular contractility, through NO production [68].

Another mediator of CC-related muscle wasting is myostatin, also known as growth differentiation factor-8 and expressed almost exclusively in skeletal muscle. Circulating levels of myostatin are increased in HF patients [69]. Myostatin binds to the activin receptor type 2B (ActRIIB) on muscle membranes, resulting in the activation of type-1 activin receptor serine kinases (ALK4 or ALK5), consequent phosphorylation of Smads 2/3 and the recruitment of Smad4 into a Smad complex. This complex translocates into the nucleus to exert changes in gene transcription, leading to muscle wasting. In addition,

myostatin binding to the receptor also reduces the kinase Akt activity and consequently diminishes FOXO phosphorylation. Dephosphorylated FOXO enters the nucleus to activate the transcription of FBXO32 and TRIM63 [70]. In contrast, insulin and IGF-1 act as suppressors of these E3 ubiquitin ligases and, therefore, have an anabolic action [71].

2.2.2 Metabolic abnormalities

The concept of metabolic failure in HF includes both impaired myocardial energy utilization and metabolic inefficiency at the systemic level. In HF patients, the global anabolic blunting and insulin resistance, together with catabolic overactivity, induce loss of skeletal muscle mass and function. The major anabolic hormones modulating protein metabolism in skeletal muscle include ghrelin, GH, insulin, IGF-1 and testosterone [7,19].

GH stimulates lipolysis, amino acids absorption, protein synthesis and glycogenolysis in the liver directly, *via* activation of tyrosine kinases, or indirectly, through induction of IGF-1 [72]. GH secretion is stimulated by increased ghrelin circulating levels, through an independent mechanism from that of hypothalamic GH-releasing hormone [33,73]. Ghrelin, the “hunger hormone”, also stimulates appetite and food intake through GH-independent mechanisms, possibly by inducing the release of neuropeptide Y (NPY) and agouti-related protein in the hypothalamus. Plus, it acts as an inhibitor of insulin and leptin signalling, as they decrease food and energy intake [74]. Despite of elevated plasma ghrelin levels in cachectic patients with HF [33], studies report that there is no appetite stimulation or weight gain in these patients, which suggest a ghrelin resistance state. The mechanism for elevated ghrelin levels in HF is still not clear, although it may be a physiological compensation for reduced weight to increase appetite and caloric intake [75,76].

Since ghrelin is responsible for GH secretion, the elevated plasma levels of ghrelin might be a direct response to GH resistance seen in HF patients. GH plasma levels are increased in CC patients when compared to non-cachectic patients and healthy control ones [30]. Changes in GH/IGF-1 axis in catabolic conditions suggest a state of GH resistance and these hormonal alterations might, in particular the low levels of IGF-1, lead to the wasting process [73]. IGF-1 mediates the effect of GH on developmental growth, stimulates cell growth and differentiation, and is a major regulator of overall metabolism. More specifically, it has been shown that IGF-1 is responsible for the stimulation of

protein synthesis and reduction of proteolysis [77]. Cachectic patients present decreased plasma levels of IGF-1 when compared to non-cachectic patients and healthy control subjects [37]. In addition, impaired glucose regulation and hyperinsulinemia are present in HF, even in the absence of diabetes mellitus, reflecting an insulin resistance state and likely contributing to disease progression. However, with the increase of HF severity and with the development of CC, circulating fasting insulin levels tend as well to decrease, for reasons that still remain unknown [78].

The steroid hormone testosterone is also another mediator involved in protein metabolism of skeletal muscle. Testosterone acts *via* intracellular androgen receptor, increasing protein synthesis by the expression of IGF-1 mRNA and downregulating IGFBP4 mRNA expression in muscle [79]. Testosterone serum levels decline during aging, and since many HF patients are elderly men with reduced exercise capacity, testosterone administration may increase skeletal muscle protein synthesis and strength, as well as improve exercise capacity in these patients [80].

Recently, there has been a growing interest in adipokines, such as leptin and adiponectin, thought to play an important role in energy metabolism and in lean/fat body mass and appetite regulation. Leptin is responsible for lipid synthesis reduction and increase of energy expenditure, inducing weight loss [81]. Circulating leptin levels are increased in HF patients when compared to healthy controls subjects. In addition, leptin levels seem to decrease in cachectic patients when compared with healthy control subjects [35,39,40]. The production of leptin may be inappropriately low in cachectic patients, which may also suggest that leptin does not contribute to the progressive worsening of CC [40,81]. Although unproven, an hypothesis that could reconcile these apparently contradictory results states that serum leptin levels increase in HF, possibly contributing to the initial catabolic process, but then decrease when CC is reached, because of the reduction in adipose tissue mass [81].

Adiponectin exerts insulin-sensitizing effects and reduces gluconeogenesis in the liver, whereas in skeletal muscle it stimulates β -oxidation [24,82]. Beyond metabolic control, adiponectin effects have been studied in the cardiovascular system: low levels are predictive of insulin resistance, atherosclerosis and inflammation, which is related to increased risk for coronary artery disease (CAD); in contrast, high adiponectin levels in healthy subjects have a protective cardiovascular effect, reducing blood pressure, total

cholesterol and LDL-C, as well as increasing insulin sensitivity [24]. Adiponectin circulating levels are increased in cachectic HF patients compared with non-cachectic ones and are also inversely correlated with BMI [24,35,41]. In addition, a study reported that patients with HF who died during a follow-up period of 51 ± 27 months, had significantly higher adiponectin levels compared to survivors [24]. Thus, increased adiponectin levels seem to be an independent predictor of mortality in patients with HF [24,41].

2.2.3 Neurohormonal abnormalities

The chronic autonomic sympathetic/parasympathetic imbalance is a crucial element of HF pathophysiology and results from general neurohormonal activation, *via* sympathetic nervous system, renin-angiotensin-aldosterone axis and natriuretic peptide system [83]. Both epinephrine and norepinephrine have been shown to cause a catabolic metabolic shift, leading to an increase in energy expenditure in cachectic HF patients [84]. Both epinephrine and norepinephrine plasma levels are increased in cachectic HF patients compared with non-cachectic ones, which suggests a specific association between cachexia and sympathetic activation in HF [30]. The consistently elevated epinephrine and norepinephrine levels lead to a sustained overstimulation of the β -adrenergic receptors signalling pathways, which ultimately diminishes β -adrenergic receptor function and impairs contractility [85].

Sustained sympathetic stimulation in HF activates the renin-angiotensin-aldosterone system and other neurohormones with subsequent salt and water retention, vasoconstriction and oedema, and is closely associated with reduced contractility and higher risk of evolving HF [86]. Increased aldosterone plasma levels and plasma renin activity, a stimulator of the production of Ang II and norepinephrine, reflect also a specific association between cachexia and neuroendocrine activation in HF [86]. Ang II induces muscle wasting through multiple mechanisms: increased oxidative stress *via* activation of NADPH oxidase; increased protein breakdown *via* reduced IGF-1 and increased cytokine signalling such as IL-6; reduced appetite *via* alteration in orexigenic/anorexigenic neuropeptide expression in the hypothalamus; impaired energy balance *via* inhibition of adenosine monophosphate-activated protein kinase; and inhibition of satellite cell function and muscle regeneration [87]. In the myocardium, Ang II leads to myocyte hypertrophy,

necrosis/apoptosis and increased collagen turnover. Also, aldosterone stimulates collagen synthesis by myocardial fibroblasts [86].

In HF, volume and/or pressure overload produces abnormal ventricular wall stress, which results on natriuretic peptide (NP) system activation, in order to restore sodium and fluid balance by natriuresis and vasodilatation. However, as the disease progresses, the functional effectiveness of the NP system becomes blunted, which contributes to worsening sodium retention and vasoconstriction, leading to further detrimental effects of the heart, with subsequent NP production [88]. In fact, both A-type NP (ANP) and B-type NP (BNP) plasma levels are increased in cachectic HF patients, in comparison with non-cachectic patients and healthy volunteers [36]. These peptides not only inhibit renin and Ang II release but also promote an increase of energy utilization and thermogenesis, by their lipolytic activities. In human fat, natriuretic peptide receptor A (NPR-A) is responsible for the activation of cGMP molecules that, subsequently, leads to protein kinase G (PKG) activation. PKG phosphorylates the hormone sensitive lipase which leads to hydrolysis of triglycerides and release of fatty acids. Thus, NP-induced lipolysis may contribute to weight loss in HF patients [89,90].

Although the molecular mechanisms underlying CC are still poorly comprehended, they are certainly multifarious involving several mediators, immunological, metabolic and neurohormonal processes [5,7]. The better understanding of these molecular pathways has been possible thanks to the development and use of animal models of CC [9].

2.3 Experimental models

Animal models represent important tools to better understand the pathogenic pathways associated with several diseases and are also crucial to find efficient therapies for these diseases [9,91]. Nevertheless, no animal model mimics exactly all features of human disease [91]. Several studies on cachexia use animal models of cancer cachexia. Since some of the underlying mechanisms of cachexia are independent of aetiology, the therapies identified for cancer cachexia might also be potentially used in CC patients but tailored therapies will surely be more effective. The development of a specific animal model for CC is essential to comprehend the molecular mechanisms at the onset of cachexia, possibly allowing an early therapeutic intervention in HF patients [9].

Recent studies have tackled the development of animal models of CC, leading to the proposal of three general groups of models: genetic, surgical and toxic models [92]. On the genetic models group, the most used animal models are the calsequestrin (CSQ)-overexpressing mice model and the Dahl salt-sensitive (DS) rats model. The first is based on the use of transgenic mice overexpressing CSQ, a calcium-binding protein of the sarcoplasmic reticulum, that develop severe cardiac hypertrophy with a significant increase in cardiomyocyte size, leading to HF [93,94]. This animal model allows to investigate not only the molecular determinants of HF but also the early skeletal muscle changes induced by HF [95]. The genetic DS rats model is based on two separated rat strains with different genetic susceptibility to develop hypertension following excessive salt ingestion [9]. The DS rats fed a high-salt diet develop hypertension and congestive HF, while DS rats fed only a low-diet salt are used as controls. This animal model presents the underlying cardiac metabolic changes on the transition of compensated left ventricle (LV) hypertrophy to congestive HF, which indicates that a metabolic remodelling of the heart might represent a therapeutic target [96]. Regarding the surgical models of CC, the most reliable ones are based on surgical methods inducing either myocardial infarction, obtained by left anterior descending (LAD) coronary artery ligation, or reduced LV output obtained through transverse aortic constriction (TAC) and ascending aortic banding [9]. Although LAD ligation technique is considered the most used animal surgical model of CC, TAC provides a more reproducible model of cardiac hypertrophy characterized by a more gradual development of HF [97]. All surgical models of CC can be used in both mice and rats; however, they are also very costly since a high mortality rate is observed [9]. One of the most commonly used and best described models of CC is the toxic model of HF and pulmonary hypertension (PH) induced by monocrotaline (MCT) [98].

2.3.1 Monocrotaline model

The MCT model is essentially a model of PH, in particular pulmonary arterial hypertension (PAH), that leads to progressive and reproducible HF and CC [9]. MCT is a macrocyclic pyrrolizidine alkaloid present in the stems, leaves and seeds of the plant *Crotalaria spectabilis*. After absorption and hepatic bioactivation, MCT cause lesions in several organs, mainly on the hepatic and cardiopulmonary system [91]. Monocrotaline pyrrole (MCTP), also named dehydromonocrotaline, is a toxic metabolite of MCT

produced in the liver by the action of the enzyme cytochrome P-450 3A and it is responsible for vascular injury and inflammation. Although the underlying mechanisms of toxicity are still unclear, it has been reported that it could result from pulmonary arterial endothelial cell damage [91,99].

The MCT animal model is based on a single MCT injection, usually of 60 mg/kg, applied intraperitoneally or subcutaneously. Although the active compound, MCTP, is degraded rapidly in aqueous solutions, such as plasma, its accumulation in erythrocytes partially explains MCT exposure effects over a time space of weeks [99,100]. Regarding the time-course of MCT effects, several studies reported signs of pulmonary vascular endothelial damage within hours after the injection. In one week, endothelial damage was reported, along with inflammatory infiltration and oedema. Two weeks after MCT injection, injected rats present an increase in pulmonary arterial pressure, leading to right ventricle (RV) hypertrophy by the third week after the injection. From this third week, rats gradually start losing weight, becoming severely cachectic and anorexic. By 5-6 weeks, half of the injected rats eventually die [9,91,98,101].

The preferred species for the study of MCT-induced CC is currently the rat. Rats are 10 times more sensitive to MCT than mice and, in addition to the difficulty associated with the image caption and catheterization, mice present less RV hypertrophy and pulmonary arterial remodelling. The response to MCT is variable among species, strains and animals because of the differences in the pharmacokinetics of MCT involving the hepatic metabolism of degradation, formation, conjugation and excretion of MCTP [9,91]. Despite this limitation, this model has been continuously used given its low cost, technical simplicity and acceptable reproducibility in comparison with other models [9].

2.4 Preventive and therapeutic approaches to cardiac cachexia

In CC-related weight loss, body wasting usually starts with the primary loss of functional muscle; therefore, early treatment approaches to increase muscle strength and exercise capacity in patients with HF are potentially more effective than strategies aimed at treating weight loss [10]. However, the results obtained so far are scarce. In addition, specific treatments towards the immune and inflammatory activation in CC have been attempted, but clinical benefits were not demonstrated [102]. Preventive and therapeutic

approaches that may potentially counteract muscle wasting or treat weight loss will be further discussed.

2.4.1 Preventive strategies for weight loss

The administration of angiotensin-converting enzyme (ACE) inhibitors and β -blockers, which are widely used in hypertension management, may potentially delay and prevent the onset of CC at early stages of HF; however, neither of them can reverse CC in HF patients [5,7]. ACE inhibitor administration can improve endothelial dysfunction, a feature of HF pathophysiology, probably through the blockade of bradykinin degradation, which stimulates the release of NO and prostaglandins [103]. ACE inhibitors are also associated with a reduction of the circulating levels of ANP, BNP, TNF- α and IL-6, as well as with a restoration of the decreased levels of IGF-1, in HF patients [5]. Moreover, treatment with the ACE inhibitor enalapril reduced the risk of weight loss in 6% or more [22]. Although therapy with high-doses of enalapril were shown to significantly decrease IL-6 activity in patients with HF, ACE inhibitors seem to have only minor influence on inflammation, since resilient immune activation is still present [104].

β -blocker administration has been included in HF treatment guidelines [5]. The sympathetic activation associated with CC can be reduced by the administration of β -blockers, which may inhibit epinephrine and norepinephrine induced lipolysis, decrease resting energy expenditure and insulin sensitivity, thus preventing weight loss and cachexia [5,105]. A study using β -blockers carvedilol and metoprolol, in HF patients with or without cachexia, showed that after 6 months of β -blocker administration, cachectic patients had an increase in body weight compared with non-cachectic ones [106]. Additionally, a study regarding the administration of the β -blocker carvedilol in HF patients, reported that the risk of death decreased in 35% when carvedilol was added to conventional therapy [107].

2.4.2 Pharmacotherapy of cardiac cachexia

As CC is a multifactorial disorder, it is unlikely that any single agent will be completely effective in treating this condition; thus, it is necessary to target different pathways [5,7]. Recent clinical trials mostly carried out in cancer patients have

investigated different approaches to regain skeletal muscle mass and strength in cachectic patients and improve survival rates [108]. There are just a few studies on drugs that counteract body wasting in HF, and CC has not specifically been targeted. Some drugs have been reported by their effects on the inhibition of particular molecules and/or proteolytic systems involved in protein catabolic pathway [109]. Potential treatments for CC and muscle wasting in HF included in the current European Society of Cardiology (ESC) guidelines are: anti-inflammatory substances, appetite stimulants, anabolic agents and exercise training, in combination with the application of nutritional supplements. However, the guidelines also warn that the safety of these treatments in HF patients is still unknown [110].

Several substances from different drug classes have been shown to suppress the production or the action of pro-inflammatory cytokines [3]. These include i) neutralizing antibodies, such as anti-TNF- α , anti-IL-1 and anti-IL-6; ii) statins, for their beneficial pleiotropic effects, namely in the reduction of pro-inflammatory cytokines and, consequently, preservation of muscle mass; and iii) other anti-inflammatory substances, such as thalidomide and pentoxifylline, which potently reduces TNF- α [3,7,109]. Preclinical studies in rats reported that etanercept, a recombinant soluble TNF receptor, may be able to reverse the deleterious negative inotropic effects of TNF- α [111]. Also, phase I clinical studies regarding etanercept treatment showed an improvement of quality of life, exercise capacity and LV ejection performance in a small number of patients with advanced HF [112,113]. Given the positive results of this clinical studies, two large clinical trials using etanercept were performed in HF patients [114]. In addition, a phase II study with infliximab, a monoclonal antibody anti-TNF, was also performed in patients with moderate to advanced HF [115]. However, in both etanercept trials and infliximab phase II study, it was observed a dose and time-dependent worsening of HF and/or worsening outcomes. Possible explanations for these results involve the intrinsic toxicity of the biological agents used in the trials, such as infliximab, which is directly cytotoxic to cells expressing TNF on the membrane, or the deleterious effects of TNF antagonism in the setting of HF, since several experimental studies suggest that physiological levels of TNF may confer cytoprotective responses in the heart during acute ischemic injury [116]. Despite the discouraging results when chronically used, a recent MCT-induced animal model of CC study showed positive results when using an anti-TNF- α treatment with

soluble TNF receptor 1 and pentoxifylline. This treatment led to an attenuation of anorexia, reduction of skeletal muscle wasting and preservation of body mass. Therefore, there is a need for more studies in animal models of CC that target inflammatory pathways [98].

Appetite stimulants have been reported to improve skeletal muscle mass and strength, appetite, caloric intake and nutritional status of cachectic patients. These include megestrol acetate, cannabinoids, ghrelin, ghrelin receptor agonists, anabolic steroids and β_2 adrenergic receptor agonists [3,5]. In these therapies, the precise mechanism by which weight gain is mediated is still unknown. Some studies suggest an increased release of NPY in healthy control rats and a modulation of calcium channels, *via* G-protein signalling, in the ventromedial nucleus of the hypothalamus of rats, commonly associated with satiety [117,118]. Also, appetite stimulants are responsible for *in vitro* reduction of pro-inflammatory cytokine production by peripheral blood mononuclear cells in cancer patients [119].

Among appetite stimulants, megestrol acetate is the most frequently used and best-studied agent, although not in patients with HF [5]. Given the many side effects associated with megestrol acetate, including thrombotic effects or even Cushing syndrome, it appears doubtful that this drug will ever be used in low-risk, stable patients with HF without extreme wasting [119]. In addition, cannabinoids are also known to stimulate appetite and increase food intake in cancer patients, but the mechanism underlying this effect is still unknown [120]. Studies *in vitro* in human cell lines suggest that they may act *via* endorphin receptors, by inhibiting prostaglandin synthesis, or may suppress cytokine production and/or secretion [121,122].

Ghrelin is not only responsible for appetite stimulation and food intake, *via* GH-independent mechanisms, but also for alterations in the cardiovascular system through inhibition of cardiomyocyte and endothelial cell apoptosis and improvement of ventricular function [6]. Nevertheless, as previously reported, plasma ghrelin levels are increased in cachectic patients with HF, regardless of BMI, despite the absence of appetite stimulation or weight gain [33,75]. One single study reported that the administration of ghrelin for 3 weeks in cachectic HF patients improved left ventricular function, exercise capacity and muscle wasting [123]. These effects may be mediated, at least in part, by GH/IGF-1 axis, which is considered to be essential for skeletal muscle metabolic homeostasis; *via* activation of NPY neurons in the hypothalamus, stimulating food intake; and by GH-

independent effects, such as vasodilation and cell apoptosis inhibition [124]. Although the precise mechanisms of these effects have not been fully identified and comprehended yet, based on these results, ghrelin or ghrelin-receptor agonists deserve further study as a therapeutic option in the CC context [6,123,124].

Anabolic steroids, including testosterone, constitute another possible therapeutic approach to treat CC since they promote protein synthesis, leading to an increase of muscle mass. The use of small doses of these substances in elderly male and female HF patients showed an improvement in cardiac function and exercise capacity, without changes in body weight [125–127]. However, the adverse side effects of the anabolic steroid administration may outweigh their potential benefits [5,7].

β_2 -adrenergic receptor agonists are known to induce skeletal muscle growth, associated with an increase in protein synthesis, a decrease in protein degradation or a combination of both. These anabolic properties make this receptor signalling pathway a novel therapeutic target for skeletal muscle wasting disorders [7,128]. However, as previously stated, the overstimulation of the β -adrenergic receptors signalling pathways seen in HF patients diminishes β -adrenergic receptor function [85]. Chronic β -adrenergic receptor stimulation can have either beneficial or detrimental effects on cardiac function depending on the pre-treatment condition of the heart [128]. A study in HF patients reported that the administration, for 12 weeks, of the β_2 -adrenergic receptor agonist clenbuterol, led to an increase in lean mass and strength, without improvement in exercise capacity. This study showed no effect on cardiac function or LV mass [129]. Newer generation of β_2 -adrenergic receptor agonists, such as formoterol, may elicit an anabolic response in skeletal muscle even at very low doses, with reduced effects on the heart and cardiovascular system [130]. Nevertheless, a better understanding of the potentially harmful cardiovascular side effects of these drugs is crucial for their application as a therapeutic approach in HF [128].

2.4.3 Nutrition

Advanced stages of HF are frequently associated with anorexia and, although CC cannot be reversed by restored nutrition alone, increases in calorie intake and protein or amino acid intake might be beneficial to regain energy reserves and consequently to increase skeletal muscle tissue, which may result in an improvement of exercise capacity

[19,46]. Some recommendations from ESC and American College of Cardiology/American Heart Association guidelines advise to limit sodium intake to 6 g/day, avoiding trigger HF decompensation by fluid retention, and to replace only detectable deficiencies of trace elements, particularly potassium, magnesium and calcium [110,131].

Nutritional interventions in patients with muscle wasting or CC have been performed using fish oils, namely omega-3 polyunsaturated fatty acid (PUFA), protein-rich, high-calorific nutritional supplements and essential amino acids [19]. A higher intake of PUFAs is correlated with a low incidence of cardiovascular disease by their anabolic and anti-inflammatory effects. For instance, dietary supplementation with PUFA significantly inhibits synthesis of TNF- α and IL-1 in severe HF patients [132]. In agreement, a study in human acute monocytic leukemia THP-1 cells reported that TNF- α production and expression induced by endotoxin LPS were significantly decreased in cells pre-incubated with PUFA [133].

Protein or amino acids supplementation might also be associated with some beneficial anabolic effects. A trial on a high-calorific protein-rich supplementation in cachectic HF patients showed an effect on the enhancement of body weight and improvement of quality of life. Additionally, it leads to a decrease in the plasma levels of TNF- α , within 6 weeks of treatment [134]. Regarding amino acid supplementation, it is known that the oral administration of essential amino acids has anabolic effects, by enhancing protein synthesis and inhibiting proteolysis. Leucine, for instance, is thought to mediate insulin signalling and glucose uptake, in skeletal muscle cells, through PI3K-AKT-mTOR pathway modulation [135]. Studies reported an improvement of peak oxygen consumption in HF and elderly HF patients by oral amino acid supplementation [136,137]. However, a recent study combining resistance exercise training and branched chain amino acid supplementation could not confirm these results. In this study, essential amino acid supplementation alone did not provide benefit to HF patients [138].

2.4.4 Exercise training and rehabilitation

According to ESC guidelines, exercise training is an established treatment for HF. Exercise training is reported to improve exercise tolerance and health-related quality of life and reduce hospitalization rates in patients with HF [19,110]. However, despite being

considered a hallmark of HF, systematic exercise intolerance studies assessing skeletal muscle function are relatively scarce [6].

Aerobic exercise training (AET), regular aerobic exercise characterized by high repetition and low resistance demands during skeletal muscle contraction, is the therapeutic strategy most verified to counteract skeletal muscle wasting in HF patients [139]. One study reported that AET significantly reduced the levels of TNF- α , IL-1 β , IL-6 and inducible nitric oxide synthase in skeletal muscle of HF patients, which suggests a reduction of local inflammation. This may represent a potential anti-catabolic intervention in HF, and therefore, an attenuation of muscle wasting [140]. More recently, a study in rats with HF induced by surgery reported that 8 weeks of AET lead to a reduction of plasma levels of TNF- α and IL-6 and an increase in the anti-inflammatory cytokine IL-10. These results suggest that AET has also an important systemic anti-inflammatory effect [141]. In addition to these effects, AET is responsible for the reduction of TRIM63 expression and myostatin mRNA levels in skeletal muscle of HF patients, not only suggesting that exercise training is effective in blocking the UPS activation related to CC but also highlighting the powerful effect of exercise in counteracting muscle wasting [142,143]. Despite the mentioned benefits of exercise training, some limitations need to be considered. Since it is common to observe fatigue in many cachectic patients, it is not expected that they would execute and conclude a AET protocol [139]. Also, the majority of elderly patients with HF are frail with multiple comorbidities, which limits the application of these protocols [144].

Currently, since none of these potential treatments is of proven benefit and their safety is unknown, further studies focused on the molecular pathways underlying CC are needed [110]. For instance, the cause of inflammatory and immune activation in HF, which plays an important role in all forms of cachexia, is still uncertain [5,7]. This lack of information makes the search for efficient therapies even more difficult. In addition, it is crucial a better comprehension of some other features of HF that may have a role on CC, clinical outcomes and survival, such as cholesterol and lipoproteins [11]. Future studies in basic and clinical science should be done in order to clarify the underlying mechanisms and to develop new strategies able to stop or even reverse the state of cachexia in HF patients and to improve quality of life and clinical outcomes [2].

3. The cholesterol paradox in heart failure

Hypercholesterolemia is an established risk factor for the development of HF, strongly associated with the presence of CAD. Cholesterol reduction therapy with statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, is effective on the reduction of morbidity and mortality in patients with CAD [12]. HMG-CoA reductase is responsible for converting HMG into mevalonate which, through several reactions, is converted to cholesterol. A significant percentage of the total cholesterol in human body is endogenously produced, by this pathway in hepatic cells, rather than up-taken by diet [145]. Blockage of cholesterol synthesis by statins is followed by an up-regulation of low density lipoprotein (LDL) receptors in the liver and by an increased uptake of LDL particles from blood [146]. However, cholesterol is a crucial molecule for the biosynthesis of steroid hormones, namely testosterone, estradiol, cortisol and aldosterone; thus, concern has been expressed regarding the potential adverse effects of long-term statin therapy [147,148]. The beneficial effects of statins are probably due not only to their LDL cholesterol-lowering effects but also to cholesterol-independent effects, including improvement in endothelial function, inhibition of neurohormonal stimulation, decrease in pro-inflammatory pathways activation and prevention of ventricular remodelling. Since CAD and HF often coexist, this therapy has become widespread use in chronic HF [149]. Paradoxically, however, low serum levels of total cholesterol and LDL-C have been reported as an adverse prognostic marker in patients with HF [11,12,14,150]. The counterintuitive phenomenon of “reverse epidemiology” is consistently reported in the catabolic stages of chronic diseases as cancer, COPD or HF [13].

The levels of circulating LDL-C can be influenced by several concomitant disorders and lifestyle interventions, such as dietary changes, reduction of excessive body weight or an increase of physical activity, as well as by the use of statins [151]. Studies have tackled the association between low levels of cholesterol/lipoproteins with worse prognosis in HF. Low LDL-C levels are associated with worse long-term outcomes and survival in patients with advanced, clinically controlled HF, particularly those on statin therapy [152,153]. High density lipoprotein cholesterol (HDL-C) levels are also a strong, independent inverse predictor of cardiovascular disease. Studies report low plasma levels of HDL-C in patients with PAH, which were associated with worse clinical outcomes [154,155]. In addition, lower levels of apolipoprotein A-I, the major component of HDL-C,

have been reported in patients with PH and are correlated with increased endothelial dysfunction [156].

Since epidemiological studies strongly support that hypercholesterolemia paradoxically improves survival in HF, a hypercaloric and cardiovascular risk-associated Western-type diet (WD) could present some benefits [157,158]. Therefore, a study tested the effect of a WD rich in saturated animal fat and simple carbohydrates, with a high salt content, on survival, PH, myocardial function, remodelling, neuroendocrine and inflammatory activity and CC in severe MCT-induced PH. This study reported that the WD ameliorated survival, PH, inflammation and CC in experimental PH [158]. The WD provides additional energy content, which is fundamental in critical illness; however, as previously reported, nutritional supplementation cannot reverse anorexia, malabsorption or catabolism [46]. Nevertheless, in this study, total cholesterol concentrations were lower in cachectic MCT-control rats and higher in MCT-WD. Moreover, NF- κ B activity and pro-inflammatory activation were reduced in the MCT-WD group. These results are concordant with the association between hypercholesterolemia and the improved survival in HF, as considered in cholesterol paradox, probably by an attenuation of immune and inflammatory activation [158].

Several hypotheses have been proposed to explain the mechanisms underlying the “reverse epidemiology” in HF, including the malnutrition-inflammation complex syndrome, or theories focusing on ubiquinone, selenoproteins or the endotoxin-lipoprotein hypothesis [14]. The malnutrition-inflammation complex syndrome hypothesis considers that low serum levels of total cholesterol are associated with low grade inflammation and protein-caloric malnutrition that leads to HF progression towards the development of CC [159]. Low cholesterol levels in patients with HF are related to both reduced BMI and serum albumin levels, the latter considered as a marker of undernutrition in patients with chronic diseases [159,160]. The ubiquinone theory states that a decrease in cholesterol is associated with a decrease in the ubiquinone production [149]. Ubiquinone, also known as coenzyme Q10 (CoQ10), uses lipoprotein-mediated transport for plasma circulation; thus, plasma levels of CoQ10 correlate with plasma total cholesterol and LDL-C levels. Along with dietary intake from meat products, CoQ10 is also synthesised endogenously by the mevalonate pathway. CoQ10, abundant in the myocardium, is not only a key component of the mitochondrial electron transport chain for ATP production, but also prevents

membrane oxidation, lipid peroxidation and promotes the recycling of α -tocopherol (also known as vitamin E) that neutralizes ROS, inducing a cardioprotective role against oxidative damage [161,162]. Therefore, myocardial CoQ10 reduction associated with low levels of cholesterol promoted by statin therapy leads to a consequent decrease of its antioxidant effects and mitochondrial ATP production, which has been postulated as a mechanism in the development and progression of HF [149,161]. The proposed theory focusing on selenoproteins also links the decreased levels of cholesterol with a decrease in antioxidant pathways [149]. Several selenoproteins are involved in protection of cells and macromolecules against oxidative stress, such as the well-known redox-active selenoenzymes: glutathione peroxidases, which represent a major class of functionally important selenoproteins, thioredoxin reductases and peptide methionine-R-sulfoxide reductase [163]. Glutathione peroxidase 4 (GPx4) catalyses the reduction of the lipid hydroperoxide to harmless alcohol and is also capable of metabolising cholesterol and cholesterol ester hydroperoxides in oxidised LDL. Lower plasma levels of cholesterol are associated with a decrease in GPx4 activity, which in turn leads to impaired prevention of LDL oxidation and subsequent uptake by endothelial cells and macrophages in arterial blood cells [164]. Ultimately, the most studied hypothesis regarding the cholesterol paradox in HF is the endotoxin-lipoprotein hypothesis [165].

3.1 The endotoxin-lipoprotein hypothesis

The endotoxin-lipoprotein hypothesis states that circulating cholesterol-rich lipoproteins and triglyceride-rich lipoproteins have the capacity to bind and detoxify bacterial LPS, by the formation of micelles. Thus, by attenuating the immune activation related to HF, cholesterol and lipoproteins may exert a protective role in HF [158,165,166]. Little is known about the physical aspects of LPS-lipoprotein interaction, although it is clearly of low affinity [167]. Lipoprotein classes, such as LDLs, very low density lipoproteins (VLDL), high density lipoproteins (HDL) and chylomicrons, have been shown to bind LPS in direct proportion to their cholesterol content [166]. In addition, for LPS concentrations relevant to human physiology, lipoproteins are an effective mechanism for LPS inactivation due to the abundance of lipoprotein particles in plasma. An *in vitro* model of endotoxaemia (i.e., presence of endotoxins in the blood) using human monocytes from healthy volunteers showed an effective inhibition of LPS bioactivity by LDL [167]. A

study *ex vivo* in chronic HF patients showed an inverse relationship between whole blood TNF- α release and serum cholesterol levels [168]. Also, incubation of macrophages with cholesterol *in vitro* led to a decrease in LPS-induced TNF- α release and mRNA expression [169].

As previously reported, immune activation seen in HF may be triggered by hypoxia, which leads to an augmented intestinal translocation of LPS into the systemic circulation, and subsequent activation of immune cells [53]. Higher plasma concentrations of LPS in HF patients are also correlated with immune activation [54]. Moreover, a study reported higher plasma LPS levels in CC. Using an *ex vivo* whole blood stimulation model, this study also showed that very small amounts of LPS are capable of inducing TNF- α secretion and soluble CD14 expression even in non-cachectic patients [170]. The cachectic ones demonstrated a reduced response after LPS stimulation, which can be explained by LPS desensitization and that may reflect previous LPS exposure *in vivo* [166,170]. The regulation of serum factors like cholesterol could be a possible explanation [166].

If the endotoxin-lipoprotein hypothesis holds true, reduction of serum lipoproteins, for instance by statin therapy, serves the wrong purpose, at least in patients with HF and possibly those with other chronic illnesses [165]. This hypothesis needs to be a matter of debate and investigation to identify suitable therapies for patients with CAD without HF and those with HF. The definition of an optimum range for serum lipoproteins and cholesterol, below which a pharmacological treatment would not be advisable in patients with CAD and HF, might be also necessary [166].

4. Aims

Given the severity of CC and the lack of therapeutic strategies, it is important to investigate its complex pathophysiology and to test new preventive and therapeutic approaches. The aim of the present work was to test the endotoxin-lipoprotein hypothesis *in vivo*, by the analysis of functional and molecular effects of dietary supplementation with cholesterol using the MCT-induced PAH, HF and CC model. We evaluated body weight evolution, effort tolerance and peak oxygen consumption, as well as cardiac function by echocardiography and haemodynamic analysis in vehicle and MCT-treated animals fed with a normal or a cholesterol supplemented diet. Plasma, cardiac and lung tissue samples were collected for molecular studies seeking to evaluate the endotoxin-lipoprotein hypothesis and to unveil the molecular and cellular mechanisms underlying the effect of cholesterol supplementation in MCT animals.

5. Material and methods

5.1 Experimental design

The experimental design is summarized in figure 2.

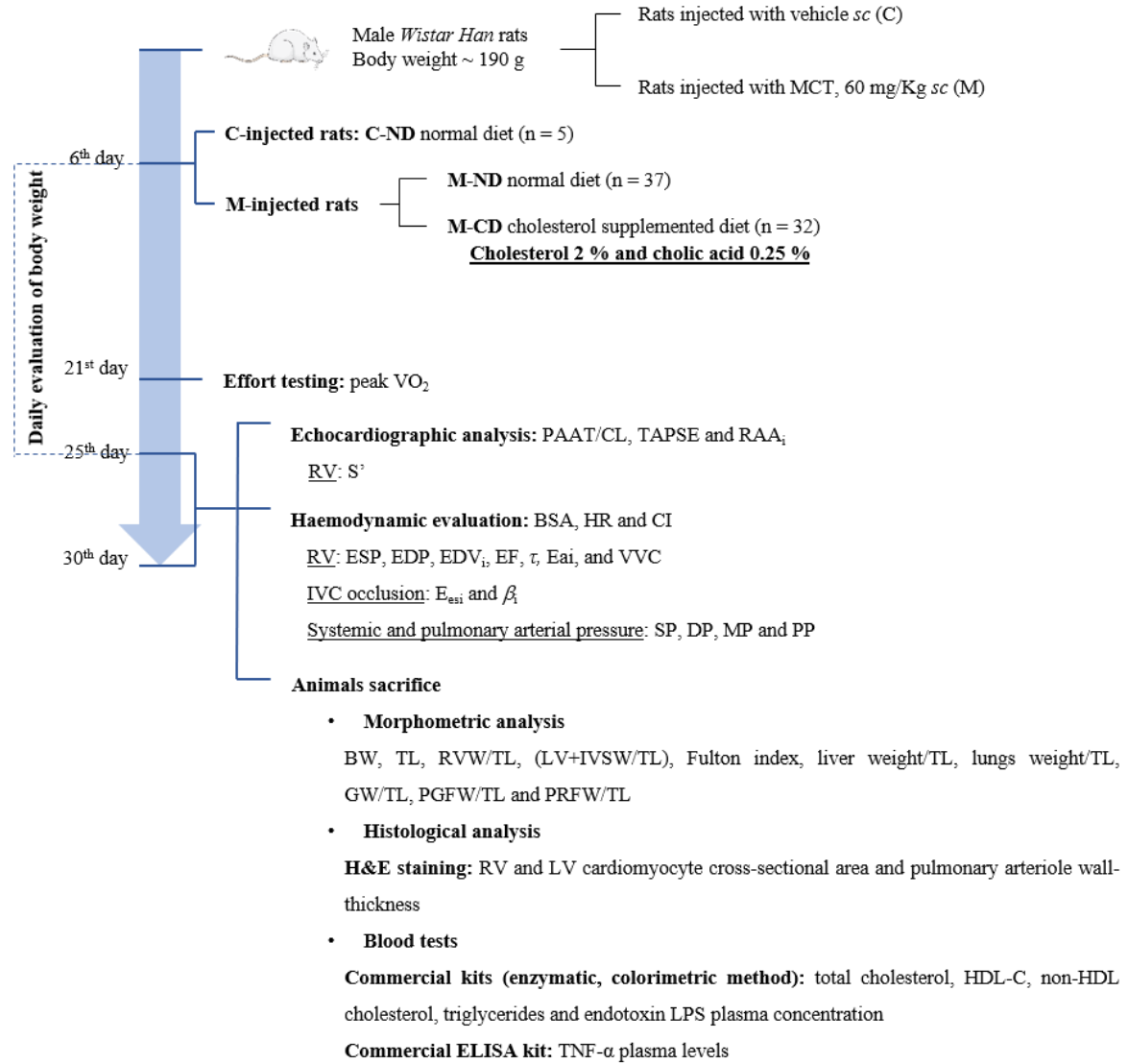


Fig. 2. Experimental protocol design. β_i , chamber stiffness constant for indexed volumes; BSA, body surface area; BW, body weight; C, control; CD, cholesterol diet; CI, cardiac index; DP, diastolic pressure; E_{ai}, arterial elastance for indexed volumes; EDP, end-diastolic pressure; EDV_i, indexed end-diastolic volume; E_{esi}, end-systolic elastance for indexed volumes; EF, ejection fraction; ELISA, enzyme-linked immunosorbent assay; ESP, end-systolic pressure; GW, *gastrocnemius* muscle weight; H&E, haematoxylin and eosin staining; HDL-C, high-density lipoprotein – cholesterol; HR, heart rate; LPS, lipopolysaccharide; LV, left ventricle; LV+IVSW, left ventricle plus interventricular septum weight; MCT and M, monocrotaline; MP, mean pressure; ND, normal diet; PAAT/CL, pulmonary artery acceleration time normalized to cycle length; PGFW, perigonadal fat weight; PP, pulse pressure; PRFW, perirenal fat weight; RAA_i, right atrial area indexed for body surface area; RV, right ventricle; RVW, right ventricle weight; S' , peak myocardial systolic velocity near the tricuspid annulus; sc, subcutaneous; SP, systolic pressure; τ , time-constant of isovolumic relaxation by

Weiss formula; TAPSE, tricuspid annular plane systolic excursion; TL, tibial length; TNF- α , tumour necrosis factor alpha; VO₂, oxygen consumption; VVC, ventricular-vascular coupling.

5.2 Animals and experimental protocol

Housing and experimental procedures were approved and complied with the Faculty of Medicine of Porto guidelines and performed in accordance with the Portuguese law on animal welfare, EU Directive 2010/63/EU, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 2011). Adult male *Wistar Han* rats (Charles River Laboratories; Barcelona, Spain) weighing 180-200 g were housed in groups of 2 *per* cage, in a controlled environment under 12h light/dark inverted cycle, at a room temperature of 22°C, with a free supply of food and water. After one week of acclimatization, rats were randomly divided in two groups, one of which received a single subcutaneous injection of MCT (60 mg/kg body weight; Sigma, Barcelona, Spain) (monocrotaline group, M) and the other an equal volume of vehicle (NaCl 0.9 %; 2 ml/kg body weight) (control group, C). Animals were fed *ad libitum* with a normal diet (ND) (4RF21A, Mucedola s.r.l.). Five days after MCT administration, rats from the monocrotaline group were randomly allocated to consume *ad libitum* either normal diet or a cholesterol supplemented diet (CD) (4RF21 based with cholesterol 2 % and cholic acid 0.25 %, Mucedola s.r.l.). Cholic acid was added to enhance cholesterol absorption [171]. Body weight (BW) was recorded daily. Twenty-one days after MCT or vehicle administration, effort testing with peak oxygen consumption (VO₂) determination was performed on a close-chamber treadmill coupled to a gas analyser at a treadmill inclination of 10°. After an initial adaption period at 15 cm/s, testing velocity was changed to 30 cm/s and then stepped up by 5 cm/s every 60 s.

5.3 Echocardiographic and haemodynamic evaluation

Since the RV hypertrophy progresses to failure around the 28th day after MCT injection [172], between the 25th and 30th day rats were anaesthetized (inhalation of 8 % sevoflurane for induction, and 2-3.5 % for maintenance) and endotracheally intubated (14G) for mechanical ventilation (150 min⁻¹, 100 % O₂, 14-16 cm H₂O inspiratory pressure, with tidal volume adjusted to animal weight, and 5 cm H₂O end-expiratory

pressure; TOPO Small Animal Ventilator - Kent Scientific, Dual Mode), and placed over a heating pad. Echocardiographic evaluation (Acuson Sequoia C512; Siemens) was performed using a 15 MHz probe (GE Healthcare) to assess tricuspid annular plane systolic excursion (TAPSE) and peak myocardial systolic velocity near the tricuspid annulus (S'), as surrogates of RV function. Right atrial area (RAAi) was also assessed. Pulmonary artery acceleration time (PAAT) was obtained from pulmonary artery flow tracings. Following echocardiographic evaluation, animals underwent haemodynamic evaluation. Under binocular surgical microscopy (Leica, Wild 384000), the right femoral vein was cannulated (24G) for fluid replacement (prewarmed 0.9 % NaCl solution, at 32 ml·kg⁻¹·h⁻¹) to compensate for perioperative losses. The heart was exposed through a median sternotomy and the pericardium was widely opened. Pressure-volume (PV) catheters were inserted through the apex in RV and LV (SPR-869 and SPR-847, respectively; Millar Instruments, Houston, TX). Another catheter (PVR-1045; Millar Instruments, Houston, TX) was inserted into the main pulmonary artery through the RV outflow tract. An ascending aorta flow probe was placed (2.5PS; Transonic, NY, USA) to allow real-time cardiac output (CO) measurement. Haemodynamic recordings were made under basal conditions with respiration suspended at end-expiration after a stabilization period of 30 minutes, and transient inferior vena cava occlusions were also obtained to derive load-independent indexes of contractility and compliance (end-systolic and end-diastolic PV relationships, respectively). Volume signal was corrected according to determination of parallel conductance by 40 µl of 10 % hypertonic saline injection, and slope factor α derived from CO measurement from the aortic flow probe. Data was continuously acquired (MPVS 300; Millar Instruments, Houston, TX), recorded at 1000 Hz (ML880 PowerLab 16/30; AD Instruments, Oxford, UK). All volumes were indexed to body surface area (BSA) as defined by $9.1 \times \text{body weight (BW, in g)}^{2/3}$ to account for differences in weights.

5.4 Morphometric evaluation

After haemodynamic assessment, animals were euthanized by exsanguination under anaesthesia. Heart, lungs, liver, *gastrocnemius* muscle, perigonadal fat and perirenal fat were excised and weighed. The RV free wall was dissected from the LV + interventricular

septum (IVS), under binocular magnification (3.5x), and weighed separately. The right tibia was also excised and its length was measured with a millimetric ruler. RV and LV + IVS, lungs, liver, *gastrocnemius*, perigonadal fat and perirenal fat weight were normalized to tibial length.

5.5 Histological analysis

For histological analysis, RV, LV and lung samples were immersion fixed in 10 % (v/v) buffered formalin by diffusion during 24 h and subsequently dehydrated with graded ethanol and included in paraffin blocks. Xylene was used in the transition between dehydration and impregnation. Serial sections (3 µm of thickness) of paraffin blocks were cut by a microtome and mounted on adhesion slides (Superfrost™ Plus, ThermoFisher Scientific, Massachusetts, USA). Deparaffinized sections were stained for haematoxylin and eosin (H&E). RV free wall specimens were obtained from each heart at midway between the apex and base. Studied samples were observed at microscope, photographed with a digital camera and measured directly at 250x magnification with a digital image analyser (cell[^]B life science basic imaging software, Olympus). Only round to ovoid nucleated myocytes were considered for analysis. Both in RV and LV samples, the cardiomyocyte cross-sectional area of sixty cardiomyocytes *per* sample was measured and averaged. On the pulmonary specimens, external diameter and medial wall thickness of pulmonary arterioles (diameter of 50 - 100 µm, 12 arterioles/lung) were analysed. Orthogonal intercepts were used to generate eight random measurements of external diameter (distance between the external lamina) and sixteen random measurements of medial thickness (distance between the internal and external lamina). For each artery, medial hypertrophy was expressed as follows: % wall thickness = $[(\text{medial thickness} \times 2)/(\text{external diameter})] \times 100$. All analyses were carried out blindly.

5.6 Blood tests

Plasma samples of vehicle or MCT-injected *Wistar Han* rats fed with a normal diet or with a cholesterol supplemented diet were analysed in duplicate to determine total cholesterol, HDL-C and triglycerides levels by enzymatic and colorimetric methods using the commercially available kits Liquick Cor-CHOL 60 2-204, PZ Cormay S.A. Lublin,

Poland; Cormay HDL 2-053, PZ Cormay S.A. Lublin, Poland and Liquick Cor-TG 60 2-253, PZ Cormay S.A. Lublin, Poland, respectively and according to the manufacturer's instructions. Non-HDL values were obtained by the difference between total cholesterol and HDL-C levels. Endotoxin LPS plasma concentration was determined in duplicate by a chromogenic method with *Limulus* amoebocyte lysate (LAL), using a commercial kit (ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit L00350, GenScript, NJ, USA) according to the manufacturer's instructions. Plasma levels of TNF- α were detected in duplicate using a commercially available enzyme-linked immunosorbent assay (ELISA) (Rat TNF- α ELISA Kit CSB-E11987r, CUSABIO, Wuhan, China) kit according to the manufacturer's instructions.

5.7 Statistical analysis

Values are given as mean \pm standard error of mean (SEM) for all variables. The Shapiro-Wilk test was performed to check normality of data. When variables were normally distributed, the statistical significance of the differences between the experimental groups was determined using one-way ANOVA, followed by the Tukey's multiple comparisons post hoc test. When the normality test failed, we performed the Kruskal-Wallis test, followed by the Dunn's multiple comparisons post hoc test. Body weight evolution was evaluated by repeated-measures one-way ANOVA. Results were considered significantly different when $p < 0.05$. Statistical analysis was performed with GraphPad Prism software (version 7.00).

6. Results

6.1 Evaluation of body weight evolution and effort tolerance with cholesterol supplemented diet in MCT-treated animals

Throughout the study, MCT-injected rats gradually developed lethargy, tachypnoea, reduced activity and signs of respiratory distress. MCT-injected animals fed with a normal diet had significantly lower body weight gain than the vehicle-injected ones ($p < 0.01$ vs. C-ND) and no significant differences were observed between M-ND and M-CD groups (Figure 3).

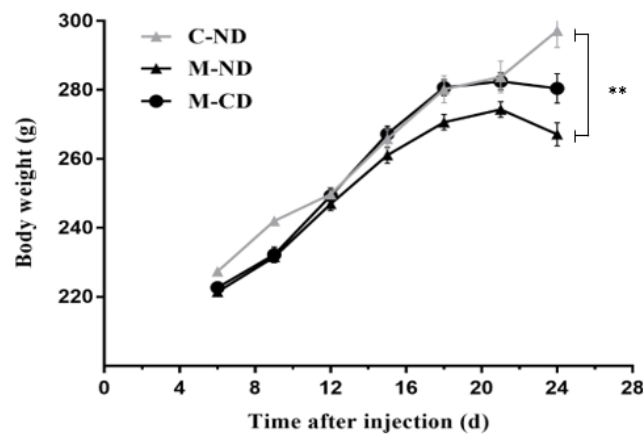


Fig. 3. Weight evolution in vehicle-injected (C-ND, $n=5$) and monocrotaline (MCT)-injected *Wistar Han* rats fed with a normal diet (M-ND, $n=37$) or with a cholesterol supplemented diet (M-CD, $n=32$). Data plotted as mean \pm SEM. Body weight was recorded from rats still surviving at each time point. ** $p < 0.01$ vs. C-ND.

Effort testing showed no significant differences between groups on peak VO_2 or total workload achieved (Figure 4).

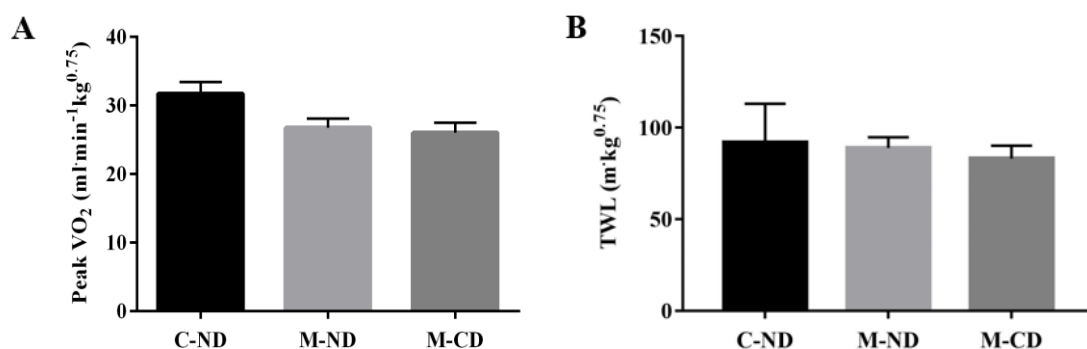


Fig. 4. Peak VO₂ (A) and total workload (B) evaluation of vehicle-injected (C-ND, n=5) and monocrotaline (MCT)-injected *Wistar Han* rats fed with a normal diet (M-ND, n=33) or with a cholesterol supplemented diet (M-CD, n=27). Bars represent mean \pm SEM. VO₂, oxygen consumption; TWL, total workload.

6.2 Analysis of echocardiographic and haemodynamic alterations associated with cholesterol supplemented diet in MCT-treated animals

RV echocardiography results are summarized in table 2.

Table 2. Echocardiographic parameters of C- or M-injected rats fed with a ND or a CD.

	C-ND	M-ND	M-CD
PAAT/CL	0.15 \pm 0.01	0.09 \pm 0.01**	0.11 \pm 0.01*
TAPSE, cm	0.27 \pm 0.01	0.21 \pm 0.01*	0.19 \pm 0.01**
RAA _i , cm ² ·m ⁻²	3.5 \pm 0.1	9.0 \pm 1.1*	7.8 \pm 1.1*
RV S', m.s ⁻¹	0.08 \pm 0.01	0.05 \pm 0.00**	0.04 \pm 0.01**

Legend: Echocardiographic data recorded between the 25th and 30th day after vehicle injection (C-ND, n=5) and monocrotaline (MCT) injection of *Wistar Han* rats, fed with a normal diet (ND) (M-ND, n=10) or with a cholesterol supplemented diet (CD) (M-CD, n=11). Values are presented as mean \pm SEM. PAAT/CL, pulmonary artery acceleration time normalized to cycle length; TAPSE, tricuspid annular plane systolic excursion; RAA_i, right atrial area indexed for body surface area; RV, right ventricle; S', peak myocardial systolic velocity near the tricuspid annulus. * p < 0.05 vs. C-ND; ** p < 0.01 vs. C-ND.

MCT-treated groups showed decreased pulmonary artery acceleration time, normalized to cycle length (p < 0.01 M-ND vs. C-ND; p < 0.05 M-CD vs. C-ND),

decreased tricuspid annular plane systolic excursion ($p < 0.05$ M-ND vs. C-ND; $p < 0.01$ M-CD vs. C-ND) and S' ($p < 0.01$ vs. C-ND) compared with C-ND group. Right atrial area, indexed for BSA, was significantly higher in both MCT-treated groups ($p < 0.05$ vs. C-ND). No differences were found between M-ND and M-CD.

Haemodynamic studies are summarized in table 3.

Table 3. Haemodynamic parameters of C- or M-injected rats fed with a ND or a CD.

	C-ND	M-ND	M-CD
Baseline			
BSA, cm^2	431 ± 7	$371 \pm 3^{****}$	$378 \pm 6^{****}$
HR, min^{-1}	342 ± 8	329 ± 11	322 ± 14
CI, $\mu L.min^{-1}.cm^{-2}$	133.1 ± 2.8	$88.4 \pm 12.1^*$	$88.0 \pm 7.6^*$
RV			
ESP, $mmHg$	29 ± 0	$61 \pm 4^{***}$	$57 \pm 4^{***}$
EDP, $mmHg$	6.4 ± 0.6	7.4 ± 0.6	6.7 ± 0.7
EDV_i, $\mu L.cm^{-2}$	0.54 ± 0.01	0.70 ± 0.19	0.58 ± 0.09
EF, %	70 ± 2	$50 \pm 6^*$	55 ± 4
τ, ms	14 ± 1	13 ± 1	12 ± 1
E_{ai}, $mmHg.\mu L^{-1}.cm^{-2}$	74 ± 3	$371 \pm 3^{****}$	$378 \pm 6^{****}$
IVC occlusion			
E_{esi}, $mmHg.\mu L^{-1}.cm^{-2}$	75 ± 6	108 ± 26	128 ± 21
β_i, $mmHg.\mu L^{-1}.cm^{-2}$	1.2 ± 0.2	$4.8 \pm 0.8^*$	$5.4 \pm 0.7^{**}$
RV VVC	1.0 ± 0.1	$0.3 \pm 0.1^{**}$	$0.3 \pm 0.0^*$

Legend: Haemodynamic data of vehicle-injected (C-ND, n=5) and monocrotaline (MCT)-injected *Wistar Han* rats, fed with a normal diet (M-ND, n=6) or with a cholesterol supplemented diet (M-CD, n=9). Values are presented as mean \pm SEM. BSA, body surface area; HR, heart rate; CI, cardiac index; RV, right ventricle; ESP, end-systolic pressure; EDP, end-diastolic pressure; EDV_i, indexed end-diastolic volume; EF, ejection fraction; τ , time-constant of isovolumic relaxation by Weiss formula; E_{ai}, arterial elastance for indexed volumes; IVC, inferior vena cava; E_{es i}, end-systolic elastance for indexed volumes; β_i , chamber stiffness constant for indexed volumes; VVC, ventricular-vascular coupling. * $p < 0.05$ vs. C-ND; ** $p < 0.01$ vs. C-ND; *** $p < 0.001$ vs. C-ND; **** $p < 0.0001$ vs. C-ND. BSA was estimated as $9.1 \cdot (\text{BW in g})^{2/3}$.

Both MCT-treated groups showed decreased BSA ($p < 0.0001$ vs. C-ND) and cardiac index ($p < 0.05$ vs. C-ND). A significant increase of right ventricular end-systolic pressure ($p < 0.001$ vs. C-ND) and pulmonary arterial elastance for indexed volumes ($p < 0.0001$ vs. C-ND) was also observed compared with C-ND. Ejection fraction was significantly lower in M-ND group ($p < 0.05$ vs. C-ND) but not in M-CD. No significant differences were observed among groups in heart rate, right ventricular end-diastolic pressure and indexed end-diastolic volume or time-constant of isovolumic relaxation. Considering the parameters evaluated from inferior vena cava occlusion, chamber stiffness constant for indexed volumes, was higher in MCT-treated groups compared with C-ND ($p < 0.05$ M-ND vs. C-ND; $p < 0.01$ M-CD vs. C-ND). No significant differences were observed among groups in end-systolic elastance for indexed volumes; however, ventricular-vascular coupling, reflected by the end-systolic elastance/pulmonary arterial elastance ratio, was significantly lower in MCT-treated animals ($p < 0.01$ M-ND vs. C-ND; $p < 0.05$ M-CD vs. C-ND).

Both M-ND and M-CD groups presented significantly lower systolic ($p < 0.01$ vs. C-ND), diastolic ($p < 0.05$ M-ND vs. C-ND; $p < 0.01$ M-CD vs. C-ND) and mean systemic arterial pressure ($p < 0.01$ vs. C-ND) compared with C-ND (Table 4). Considering pulmonary arterial pressure, MCT-treated animals presented significantly higher values of systolic ($p < 0.001$ M-ND vs. C-ND; $p < 0.0001$ M-CD vs. C-ND), diastolic ($p < 0.01$ M-ND vs. C-ND; $p < 0.05$ M-CD vs. C-ND) and mean pressure ($p < 0.0001$ M-ND vs. C-ND; $p < 0.001$ M-CD vs. C-ND). Additionally, MCT-injected animals also had an increased pulmonary arterial pulse pressure when compared to vehicle-injected ones ($p < 0.05$ M-ND vs. C-ND; $p < 0.001$ M-CD vs. C-ND).

Table 4. Systemic and pulmonary arterial pressure of C- or M-injected rats fed with a ND or a CD.

	C-ND	M-ND	M-CD
Systemic arterial pressure			
SP, mmHg	122 ± 4	95 ± 7**	95 ± 4**
DP, mmHg	90 ± 2	57 ± 8*	53 ± 7**
MP, mmHg	107 ± 2	75 ± 9**	76 ± 5**
PP, mmHg	31 ± 2	37 ± 3	47 ± 10
Pulmonary arterial pressure			
SP, mmHg	32 ± 0	63 ± 5***	62 ± 3****
DP, mmHg	16 ± 1	31 ± 3**	25 ± 2*
MP, mmHg	24 ± 1	44 ± 3****	41 ± 2***
PP, mmHg	16 ± 1	32 ± 3*	37 ± 3***

Legend: Systemic and pulmonary arterial pressure data of vehicle-injected (C-ND, n=5) and monocrotaline (MCT)-injected *Wistar Han* rats, fed with a normal diet (M-ND, n=6) or with a cholesterol supplemented diet (M-CD, n=9). Values are presented as mean ± SEM. SP, systolic pressure; DP, diastolic pressure; MP, mean pressure; PP, pulse pressure. * $p < 0.05$ vs. C-ND; ** $p < 0.01$ vs. C-ND; *** $p < 0.001$ vs. C-ND; **** $p < 0.0001$ vs. C-ND.

6.3 Morphometric alterations associated with cholesterol supplemented diet in MCT-treated animals

Results of morphometric evaluation are shown in table 5.

Table 5. Morphometric parameters of C- or M-injected rats fed with a ND or a CD.

	C-ND	M-ND	M-CD
Body weight, g	326 ± 8	255 ± 6****	265 ± 6****
TL, mm	39 ± 0	38 ± 0	38 ± 0
RVW/TL, mg.mm⁻¹	4.3 ± 0.1	8.1 ± 0.5**	9.5 ± 0.6****
(LV+IVSW)/TL, mg.mm⁻¹	16 ± 0	15 ± 0	16 ± 1
Fulton index, g.g⁻¹	0.27 ± 0.01	0.53 ± 0.04*	0.59 ± 0.04***
Liver weight/TL, mg.mm⁻¹	265 ± 9	254 ± 17	327 ± 14††
Lungs weight/TL, mg.mm⁻¹	37 ± 1	65 ± 5*	74 ± 6**
GW/TL, mg.mm⁻¹	54 ± 1	44 ± 2**	48 ± 1
PGFW/TL, mg.mm⁻¹	65 ± 3	55 ± 4	47 ± 4*
PRFW/TL, mg.mm⁻¹	56 ± 2	53 ± 6	39 ± 3

Legend: Morphometric data of vehicle-injected (C-ND, n=5) and monocrotaline (MCT)-injected *Wistar Han* rats, fed with a normal diet (M-ND, n=11) or with a cholesterol supplemented diet (M-CD, n=11). Values are presented as mean ± SEM. TL, tibial length; RVW, right ventricle weight; LV+IVSW, left ventricle plus interventricular septum weight; GW, *gastrocnemius* muscle weight; PGF, perigonadal fat weight; PRF, perirenal fat weight. * $p < 0.05$ vs. C-ND; ** $p < 0.01$ vs. C-ND; *** $p < 0.001$ vs. C-ND; **** $p < 0.0001$ vs. C-ND; †† $p < 0.01$ vs. M-ND.

Body weight decrease in MCT-injected animals occurred simultaneously with a significant decrease in *gastrocnemius* muscle weight/tibial length ratio only in M-ND group ($p < 0.01$ vs. C-ND group) but not in M-CD. The development of cardiac hypertrophy was also present in M-ND and M-CD groups, as evidenced by the significant increase in right ventricle weight/tibial length ratio ($p < 0.01$ M-ND vs. C-ND group; $p < 0.0001$ M-CD vs. C-ND group) and Fulton index ($p < 0.05$ M-ND vs. C-ND group; $p < 0.001$ M-CD vs. C-ND group). MCT-treated animals also presented a significantly increase in lung weight/tibial length ratio ($p < 0.05$ M-ND vs. C-ND group; $p < 0.01$ M-CD vs. C-ND group). Cholesterol supplemented diet induced a significant increase in liver weight/tibial length ratio in MCT-treated animals ($p < 0.01$ vs. M-ND group). Furthermore,

M-CD group showed a significantly lower perigonadal fat weight/tibial length ratio when compared with C-ND ($p < 0.05$ vs. C-ND group). No significant differences were observed between groups in tibial length, left ventricle plus interventricular septum weight/tibial length ratio or perirenal fat weight/tibial length ratio.

6.4 Evaluation of histological alterations associated with cholesterol supplemented diet in MCT-treated animals

Illustrative histological sections of RV and LV cardiomyocyte cross-sectional area are presented in figure 5.

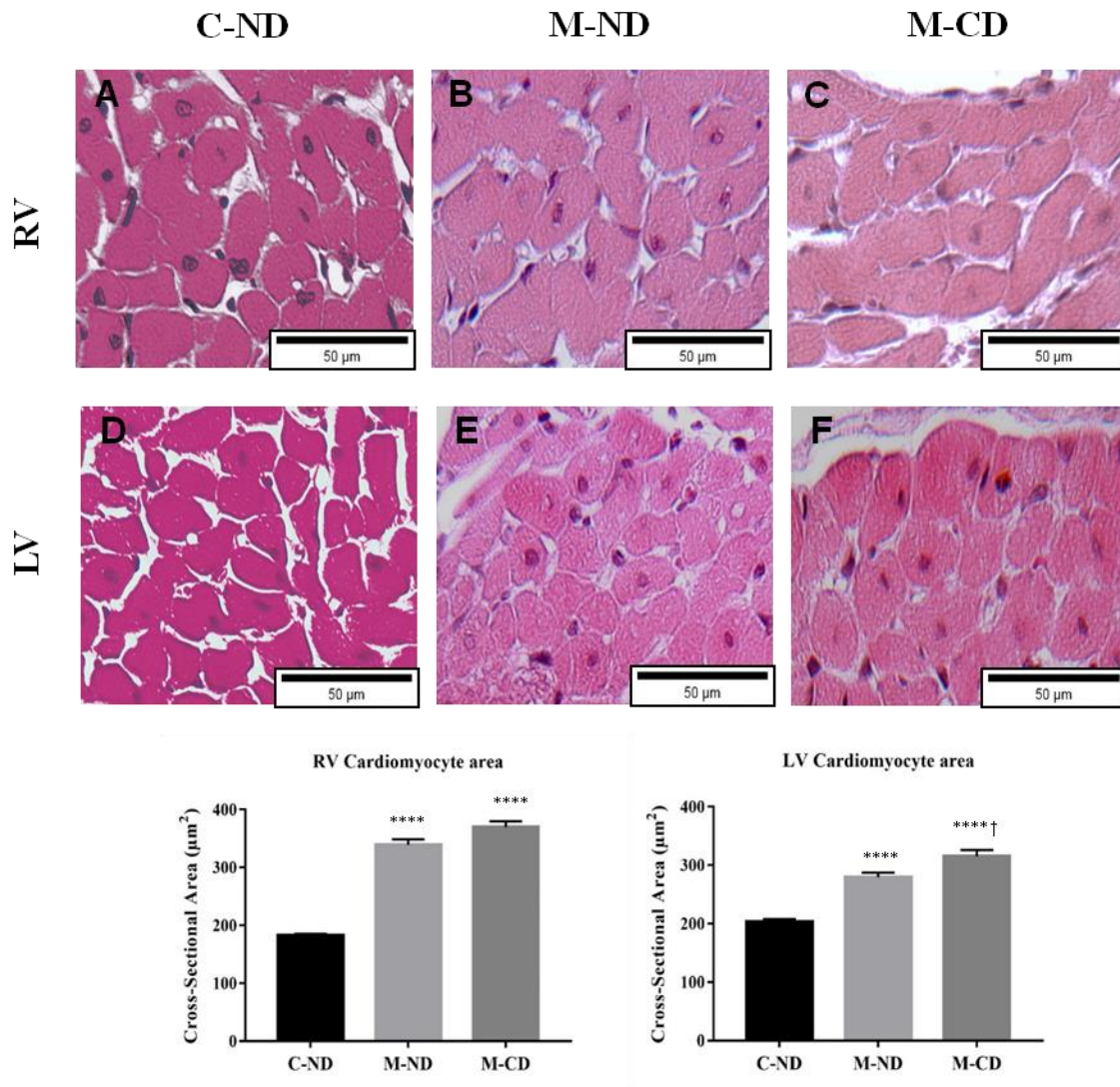


Fig. 5. Representative H&E staining of RV (Panels A, B and C) and LV (Panels D, E and F) cardiomyocyte cross-sectional area of vehicle-injected (C-ND, n=5) and monocrotaline (MCT)-injected *Wistar Han* rats, fed with a normal diet (M-ND, n=11) or with a cholesterol supplemented diet (M-CD, n=10). Bars represent mean \pm SEM. RV, right ventricle; LV, left ventricle. **** $p < 0.0001$ vs. C-ND; † $p < 0.05$ vs. M-ND.

MCT-treated animals had higher RV and LV cardiomyocyte cross-sectional area ($p < 0.0001$ vs. C-ND group). Cholesterol supplemented diet increased LV cardiomyocyte cross-sectional area ($p < 0.05$ vs. M-ND group).

Illustrative histological sections of lung arterioles are presented in figure 6.

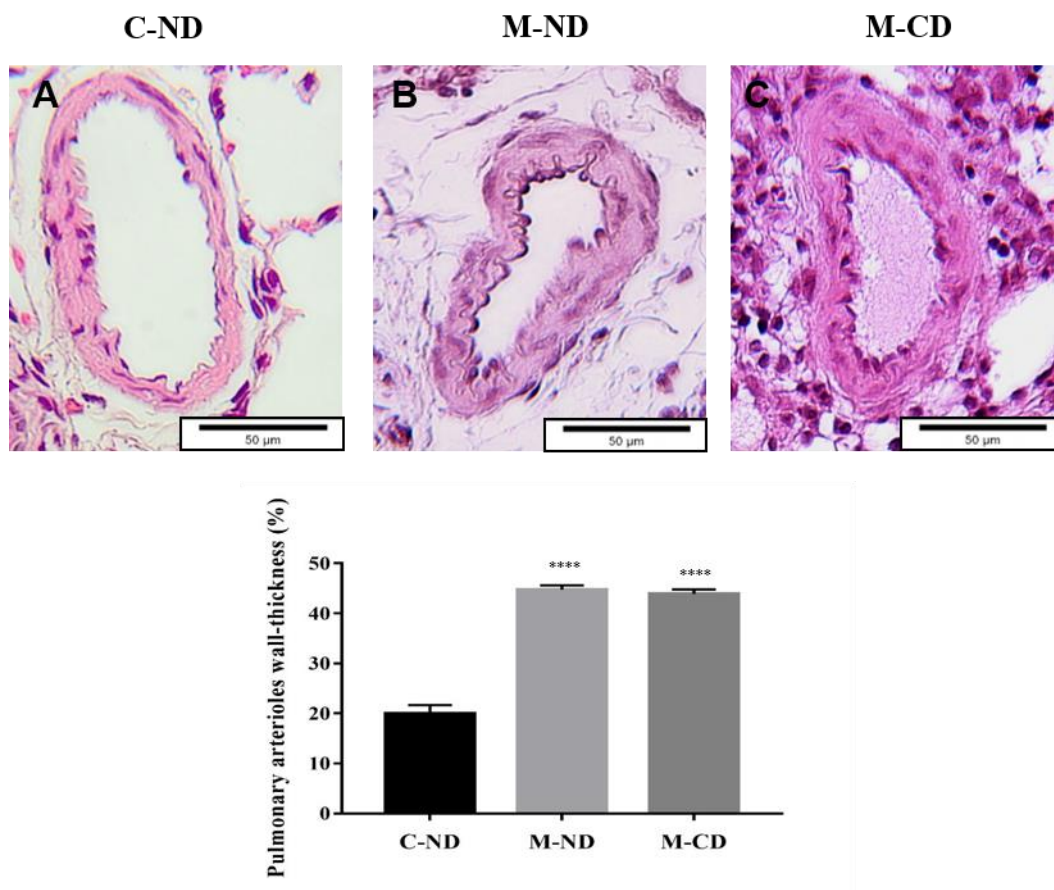


Fig. 6. Representative H&E staining of lung arterioles in vehicle-injected (C-ND, n=5) (Panel A) and monocrotaline (MCT)-injected *Wistar Han* rats, fed with a normal diet (M-ND, n=6) (Panel B) or with a cholesterol supplemented diet (M-CD, n=7) (Panel C). Bars represent mean \pm SEM. **** $p < 0.0001$ vs. C-ND.

Significant increase of pulmonary arteriole wall-thickness was observed in MCT-treated animals compared with C-ND ($p < 0.0001$ vs. C-ND group).

6.5 Determination of total cholesterol, HDL-C, non-HDL cholesterol and triglycerides plasma concentration in MCT-treated animals fed with a cholesterol supplemented diet

Plasma total cholesterol, HDL-C, non-HDL cholesterol and triglycerides levels are reported in table 6.

Table 6. Plasma concentration of total cholesterol, HDL-cholesterol, non-HDL cholesterol and triglycerides from C- or M-injected rats fed with a ND or a CD.

	C-ND	M-ND	M-CD
Total cholesterol, $mg.dl^{-1}$	20.68 \pm 1.34	23.24 \pm 4.26	74.43 \pm 14.42 ^{***†}
HDL-C, $mg.dl^{-1}$	20.23 \pm 1.69	18.64 \pm 3.52	39.96 \pm 7.83 [†]
Non-HDL cholesterol, $mg.dl^{-1}$	0.45 \pm 0.40	4.60 \pm 1.27	34.47 \pm 6.82 ^{**}
TG, $mg.dl^{-1}$	124.44 \pm 20.16	34.41 \pm 6.99 ^{**}	50.44 \pm 13.39 ^{**}

Legend: Plasma concentration of total cholesterol, HDL-cholesterol, non-HDL cholesterol and triglycerides of vehicle-injected (C-ND, n=5) and monocrotaline (MCT)-injected *Wistar Han* rats, fed with a normal diet (M-ND, n=5) or with a cholesterol supplemented diet (M-CD, n=5). Values are presented as mean \pm SEM. HDL-C, high density lipoprotein – cholesterol; TG, triglycerides. * $p < 0.05$ vs. C-ND; ** $p < 0.01$ vs. C-ND; † $p < 0.05$ vs. M-ND; †† $p < 0.01$ vs. M-ND.

No significant differences were observed between M-ND and C-ND groups in total cholesterol, HDL-C or non-HDL cholesterol plasma concentration. Triglyceride plasma concentration was lower in MCT-treated animals when compared to vehicle-injected ones ($p < 0.01$ vs. C-ND group). Cholesterol supplemented diet induced a significant increase in total cholesterol ($p < 0.01$ vs. M-ND group) and HDL-C plasma concentration ($p < 0.05$ vs. M-ND group) in MCT-treated animals. M-CD group showed significantly higher non-HDL cholesterol plasma concentration compared with C-ND ($p < 0.01$ vs. C-ND group).

6.6 Evaluation of endotoxin LPS plasma levels in MCT-treated animals fed with a cholesterol supplemented diet

In order to evaluate the endotoxin-lipoprotein hypothesis, we determined endotoxin LPS plasma concentration. Results are shown in figure 7.

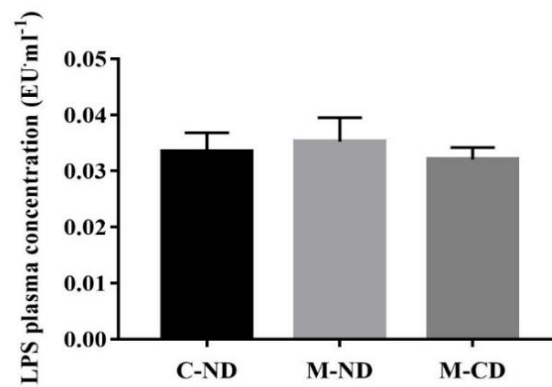


Fig. 7. Plasma concentration of LPS in vehicle-injected (C-ND, n=5) and monocrotaline (MCT)-injected *Wistar Han* rats, fed with a normal diet (M-ND, n=10) or with a cholesterol supplemented diet (M-CD, n=10). Bars represent mean \pm SEM. LPS, lipopolysaccharide.

The results obtained in the quantification of LPS plasma concentration show no significant differences between groups.

6.7 Determination of TNF- α plasma levels in MCT-treated animals fed with a cholesterol supplemented diet

Plasma TNF- α levels are reported in figure 8.

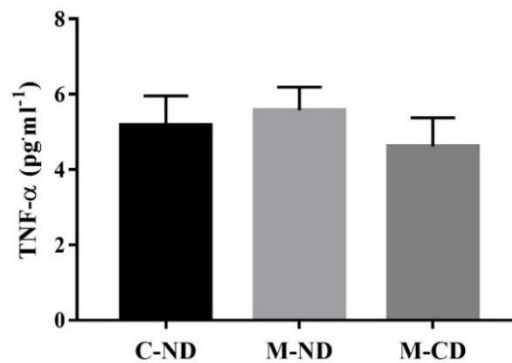


Fig. 8. Plasma concentration of TNF- α in vehicle-injected (C-ND, n=5) and monocrotaline (MCT)-injected *Wistar Han* rats, fed with a normal diet (M-ND, n=9) or with a cholesterol supplemented diet (M-CD, n=8). Bars represent mean \pm SEM. TNF- α , tumour necrosis factor alpha.

The results obtained in the quantification of TNF- α plasma levels show no significant differences between groups.

7. Discussion

Since none of the various explored therapeutic approaches targeting CC so far proved to have beneficial effects and their safety remains an issue, clinicians are currently only endorsed to manage underlying disease and comorbidities of CC, which is notoriously inefficient [102,110]. Therefore, further studies are warranted to unravel CC pathophysiology and new therapeutic strategies [10]. Following up on cholesterol paradox, the puzzling finding that low cholesterol and LDL levels worsen prognosis in HF [11], and taking into account the endotoxin-lipoprotein hypothesis as a likely explanation [166], we hypothesized that enhancing cholesterol intake would protect animals with HF and CC. In this work, we newly put to test the endotoxin-lipoprotein hypothesis in experimental CC associated with HF and PAH *in vivo*, by evaluating functional and molecular effects of a diet rich in cholesterol in MCT-induced rat PAH. This is a widely used animal model for the study of the pathogenesis of PAH, associated with progressive HF and CC. MCT-induced PAH animal model is characterized by remodelling and occlusion of the pulmonary arterial vessels, leading to progressively higher vascular resistance and RV afterload, development of RV dysfunction and failure [99,173]. Although there are some differences described between this animal model and human PAH, such as the absence of plexiform lesions in the experimental model, it has identical haemodynamic and morphological features and high mortality [91,174]. Indeed, as expected, we observed a high mortality rate in MCT-treated rats. The PAH model was successfully established, as mean pulmonary arterial pressure was above 25 mmHg and significantly increased in the MCT-treated rats, which is in accordance with the observed in human PAH [175]. Pulmonary vascular remodelling was evident on histological evaluation and by the increase in pulmonary arteriole wall-thickness. In addition, pulmonary oedema and vascular engorgement was also present in MCT-treated animals, as quantified by the lung weight/tibial length ratio. In MCT-injected rats, the RV was submitted to an increased afterload, as shown by the higher pulmonary arterial elastance. To counteract the pressure overload, RV underwent a process of remodelling with an initial adaptive response of compensatory hypertrophy (concentric remodelling). Hypertrophic response was evident by the significant increase of RV weight, Fulton index, cardiomyocyte hypertrophy, right ventricular end-systolic pressure and higher intrinsic myocardial contractility, observed by the increase of the load-independent contractility index end-systolic elastance. In addition,

ventricular-vascular coupling efficiency was decreased in MCT animals, which probably resulted of an insufficient increase in contractility to counteract the increased afterload [176,177]. This abnormal ventricular-vascular coupling, along with decreased right ventricular ejection fraction, is a relevant feature of RV failure [178,179]. Indeed, decreased cardiac index in MCT-injected rats, mostly due to impaired stroke volume in MCT-injected rats corroborates the HF state [180]. Other signs of RV failure were present, such as an upward shift in the end-diastolic pressure volume relationship (β_i) related to an increased chamber stiffness which can possibly be explained by fibrosis deposition, as reported in previous reports [158,181]. The low cardiac output state and ventricular interdependence also explain systemic hypotension, as assessed by systolic, diastolic and mean systemic arterial pressures [180]. Despite this, in our evaluation there was no evident RV dilatation suggesting that in most animals there was still no evolution to overt RV failure. In fact, it is expected that with sustained pressure overload animals progress to maladaptive RV hypertrophy (eccentric remodelling) with RV dilatation [158,182–184]. Although we found lower BW in MCT-treated animals, accompanied by decreased *gastrocnemius* muscle weight, which is suggestive of muscle wasting related to CC and consistent with previous reports [177,181,185], we must underscore that previous works from ours and other groups have shown that further BW loss and muscle wasting would be expected at a later stage of disease. The lack of progression to full-blown RV failure and CC may explain the absence of differences in effort tolerance, either peak VO_2 or total workload achieved. Most likely our evaluation was carried out at an early stage of disease evolution where most animals were still not in severe HF, indeed, when MCT-injected animals are evaluated at latter stages of disease impaired exercise capacity and decreased peak VO_2 are expected [176].

In this animal model of PAH, MCT is responsible for endothelial cell injury and subsequent massive mononuclear infiltration into the perivascular regions of arterioles and muscular arteries, thus leading to vascular remodelling [175,186]. This inflammatory activation is a central player in PAH development, leading to a systemic inflammatory state [181]. In fact, several pro-inflammatory cytokines, such as $\text{TNF-}\alpha$ and IL-6, are highly expressed both in human PAH [187–189] and animal models of PAH [177,181]. We found a trend towards increased $\text{TNF-}\alpha$ plasma levels in MCT-treated animals,

suggesting that sampling at more advanced stages of disease would probably reveal higher TNF- α levels in MCT-injected animals.

Even though hypercholesterolemia is an established cardiovascular risk factor for the development of CAD, which often coexists with HF, low total cholesterol and LDL-C levels are associated with poor prognosis in patients with severe HF [12]. In order to explain the paradoxical relationship between cholesterol levels and survival in HF, the endotoxin-lipoprotein hypothesis states that circulating lipoproteins have the capacity to bind and detoxify bacterial LPS [165,166]. If this hypothesis holds true, higher levels of cholesterol and lipoproteins would be expected to attenuate the immune activation related to HF [190]. We evaluated the effects of a cholesterol rich diet in the MCT-induced PAH, HF and CC model. Based on previous reports, we used a diet supplemented with cholesterol 2 % and cholic acid 0.25 % in order to induce a hypercholesterolaemic state [191]. In fact, our results showed a significant increase of total cholesterol, HDL-C, non-HDL cholesterol and triglycerides plasma concentration in MCT-treated animals fed with this cholesterol rich diet. Although non-significantly, cholesterol supplemented diet induced an increase in BW and *gastrocnemius* muscle weight of the MCT-treated animals, which is suggestive of an amelioration of the muscle wasting related to CC. These results were also observed on a previous study regarding the effects of a WD rich in saturated animal fat and simple carbohydrates, with a high salt content, on MCT-induced PH with CC [158]. Cholesterol supplemented diet in MCT-injected rats also showed a trend towards decreased TNF- α plasma levels, which suggests the potential to ameliorate systemic inflammation in CC. This result is consistent with the reduction of NF- κ B activity and consequent decrease of cytokine activation observed in the previous report [158]. Nevertheless, no major changes were found in pulmonary hypertension, cardiac remodelling and function between MCT-injected rats regardless of diet type, except for trends towards lesser RV dilatation and improved ejection fraction. Of note, despite the short course of hypercholesterolemic diet, animals already showed LV cardiomyocyte hypertrophy previously related to renin-angiotensin-aldosterone system activation [192] and increased liver weight typical of steatosis [193]. Regarding the endotoxin-lipoprotein hypothesis, to put this hypothesis to test we were expecting total cholesterol reduction in MCT-injected rats fed with a normal diet compared to healthy rats [158]. Nevertheless, possibly due to the fact that animals were evaluated at an earlier time-point when CC was

not that pronounced in most of the animals, we did not reproduce these findings. We found higher plasmatic concentration of total cholesterol and non-HDL cholesterol in MCT-treated animals fed with a cholesterol diet as well as trend towards lower plasma levels of endotoxin LPS, suggesting that the higher lipoprotein content might have a beneficial role in lowering LPS induced pro-inflammatory effects, as proposed in previous reports [134,158].

We must underscore that although many similarities remain, rats and humans have important differences in cholesterol metabolism, such as the absence of cholesterol ester transfer protein in rats, which restricts the transport and clearance of cholesterol esters to the HDL compartment, along with rapid clearance of VLDL and chylomicrons by the liver, which substantially reduces circulating LDL levels [194,195].

8. Conclusions

In the present work, we aimed to test the endotoxin-lipoprotein hypothesis *in vivo* in the MCT-induced rat PAH, HF and CC model and evaluated the functional and molecular effects of a standard cholesterol-rich diet. Our results suggest that there may be a role for cholesterol supplementation in attenuating the systemic inflammation associated with CC in PAH and HF, although no definitive conclusions can be taken. Unfortunately, our timing of evaluation did not allow us to observe severe inflammatory activation, HF and CC in MCT-injected animals and we found large variability within groups; therefore, the role of cholesterol supplementation could not be fully appraised. Another concern was the dose selected for cholesterol supplementation. We based our intervention in previous studies carried out to induce hypercholesterolemia [191] but, in our particular case, it is likely that lower doses might have been more effective. Indeed, hypercholesterolemia has unwanted effects such as inflammation itself and neurohumoral changes in renin-angiotensin-aldosterone system [192] that may have taken place according to our data on LV hypertrophy.

Since the results obtained were showed to be promising and revelling that cholesterol supplementation might have some beneficial effects in attenuating the inflammatory response induced by endotoxin LPS, future studies are needed in order to explore this issue. As future perspective, our work encourages more research in cholesterol supplementation in MCT-induced CC using lower doses of cholesterol, at more severe stages of disease or in other models of CC. Moreover, we know that due to its complex pathophysiology, a therapeutic strategy targeting CC should combine different agents [5]. Our study supports the addition of other interventions such as appetite stimulants or other nutritional supplements to dietary cholesterol supplementation [19,119].

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